IMPACT OF ETHNICITY AND FTO (FAT MASS AND OBESITY ASSOCIATED GENE) ON ENERGY EXPENDITURE AND FUEL UTILIZATION

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Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Song Lin Lin, Tammy

23rd January 2015

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Great is Thy faithfulness
Great is Thy faithfulness
Morning by morning new mercies I see
All I have needed Thy hand hath provided
Great is Thy faithfulness, Lord, unto me

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Summary

Background

There is an exponential rise in the prevalence of obesity in Singapore coincident with the rapid nutritional and socio-economic transition. Significant ethnic differences in obesity predisposition exist. Reduced resting energy expenditure (REE) and metabolic flexibility (MF) in substrate switching may be contributing factors. Multiple single nucleotide polymorphisms (SNPs) in the first intron of FTO (fat mass and obesity associated) gene are found to be associated with an increase in body mass index (BMI), however the underlying mechanisms by which FTO influences adiposity are still unexplored.

Objectives

This thesis aims to

1) Evaluate differences in REE and MF among Chinese, Malay and Indian men.
2) Characterize the function of FTO in skeletal muscle metabolism and energy homeostasis.

Methods

268 Chinese, Malay and Indian men aged 21-40 years old, BMI of 18.5 to 30.0 kg/m^2 and have sedentary lifestyles were recruited in this cross sectional study. Body composition was measured by dual energy X-ray absorptiometry. Energy expenditure and respiratory quotient (RQ) were assessed by indirect calorimetry

at fasting and during a mixed meal tolerance test. FTO function was explored using two approaches a) genetic approach by gene silencing and b) chemical probe approach using a novel pharmacologic inhibitor (CA) of FTO m^6A demethylase activity

Results

1) After adjustment for body weight, REE was significantly lower (73±22 kcal/day) in Asian-Indians than in Chinese. The association between REE and ethnicity was no longer statistically significant after total FFM was replaced by trunk FFM (which includes heart, liver, kidney and spleen) but not when it was replaced by limb FFM (skeletal muscle). Among lean subjects, the RQ (iAUC) was significantly lower in Chinese (0.010 ± 0.004) compared with Asian-Indians (0.035±0.005, P<0.001) or Malays (0.037±0.005, P<0.001). Among overweight/obese subjects, there were no ethnic differences observed for RQ (iAUC).
2) FTO knockdown and CA treatment in primary human myotubes induced gene expression of UCP3, CPT1B, HADHA, ATP5J2 and PGC1A but downregulated gene expression of ACC1 and FASN. In CA treated myotubes, a significant increase in mitochondrial respiration was observed, in particular spare respiratory capacity. CA was also able to alleviate the reduction in glucose uptake caused by palmitate acid.

Conclusions

We have found that Asian Indian men exhibit lower REE than Chinese men for the same body weight, which may contribute to the higher prevalence of obesity in this ethnic group. Lower REE in Asian-Indian men may be mediated by smaller size of high metabolic rate organs in the trunk and the brain. To prevent obesity in Asian-Indians, the recommended dietary allowance for energy intake should take into account the lower REE in this ethnic group. In addition, the relationship between MF and obesity is modulated by ethnicity. In lean individuals, Chinese had lower MF than Malays and Indians. However, in overweight/obese individuals, MF was similar between ethnic groups.

Inhibition of FTO catalytic activity by CA increased fatty acid oxidation, and decreased de novo lipogenesis in human skeletal muscle. CA also improved mitochondrial oxidative metabolism and basal glucose uptake. Collectively, these findings suggest that pharmacologic inhibition of FTO could be a viable therapeutic strategy for obesity.

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List of Abbreviations

ACC1	Acetyl-CoA Carboxylase 1
AMPK	AMP-activated protein kinase
ANT	Adenine nucleotide translocase
ATM	Adipose tissue macrophages
ATP5J2	ATP Synthase
ATPase	Adenosinetriphosphatase
BAT	Brown adipose tissue
BIA	Bioelectrical impedence analysis
BMC	Bone mineral content
BMI	Body mass index
CAT	Carboxyatractylate
CHO	Carbohydrate
CS	Citrate synthase
COX15	Cytochrome c oxidase
CPT1B	Carnitine palmitoyltransferase 1B
DAG	Diacylglycerol
DEXA	Dual energy X ray absorptiometry
DIO	Diet induced obese
DMEM	Dulbecco's modified Eagle's medium
ETC	Electron transport chain
eTIV	Estimated total intracranial volume
FASN	Fatty acid synthase
FAO/WHO/UNU	Food and Agriculture Organization/World Health Organization/United Nations University
FADH2	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide p-trifluoro methoxyphenylhydraz
FGF21	Fibroblast growth factor 21
FFM	Fat-free mass
FTO	Fast mass and obesity associated
GWAS	Genome wide association studies
HADHA	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase
HMRO	High metabolic rate organs
HS	Horse serum

IDH3G	Isocitrate dehydrogenase 3 (NAD+) gamma
IMCL	Intramyocellular fat
IL-6	Interleukin-6
ISI	Insulin sensitivity index
LBM	Lean body mass
MCP1	Monocyte chemoattractant protein 1
MC4R	Melanocortin 4 receptor
MF	Metabolic flexibility
MMTT	Mixed meal tolerance test
m6A	N6-methyladenosine
NADH	Nicotinamide adenine dinucleotide
OCR	Oxygen consumption rate
PAI-1	Plasminogen activator inhibitor-1
PCr	Phosphocreatinine
PGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PFK	Phosphofructokinase
PI3K	Phosphatidylinositol 3-kinase
POMC	Pro-opiomelanocortin
PS	Penicillin-streptomycin
REE	Resting energy expenditure)
RQ	Respiratory quotient
SAT	Subcutaneous abdominal adipose tissue
SMR	Sleeping metabolic rate
TCA	Tricarboxylic acid cycle
TEE	Total energy expenditure
T2D	Type 2 diabetes
UCP	Uncoupling protein
VAT	Visceral abdominal adipose tissue
VO_2	Volume of oxygen consumption
VCO_2	Volume of carbon dioxide production
WHO	World Health Organization

Chapter 1 : Introduction and Literature Review

1.1 Obesity

According to the World Health Organization, more than 1 billion adults are overweight (body mass index (BMI)>25 kg/m^2) and more than 300 million are obese (BMI>30 kg/m^2); these numbers are expected to double by the year 2025(3). Obesity represents a significant risk factor for mortality and many medical conditions including cardiovascular disease, stroke, hypertension; dyslipidemia, type 2 diabetes (T2D), osteoarthritis, asthma, non-alcoholic liver disease, gallstones and certain cancers(4). In Singapore, obesity is also reaching epidemic proportions in coincident with rapid nutritional and socioeconomic transition. The prevalence of obesity (BMI >30 kg/m^2) had increased from 6.9% in 2004 to 10.8% in 2010. By BMI risk categories created for Asian populations including Singapore, 23.0% were in the high risk BMI category. Among the ethnic groups, Malays had the highest proportion of obese adults (24.0%) followed by Asian-Indians (16.9%) and Chinese (7.9%)(5). Despite the urgent need for therapeutic intervention, there is currently no safe and effective treatment for the disease.

1.2 Causes of obesity

The pathophysiology of obesity is characterized by a chronic energy imbalance between caloric intake and energy expenditure, however the relative contribution of inter-individual differences in energy intake and expenditure on this balance is unclear(6). Positive energy balance occurs when excessive overfeeding relative to energy expenditure occurs, and the

body increases its overall energy stores. Besides energy balance, macronutrient intake must also balance macronutrient oxidation. Even after controlling for energy balance and body fat, a positive association between 24-h respiratory quotient (RQ)(high ratio of carbohydrate to fat oxidation) and weight gain was observed (7, 8). Higher carbohydrate oxidation can reduce glycogen stores and increase energy intake(9).

1.3 Resting energy expenditure

1.3.1 Definition

Resting energy expenditure (REE) or basal/standard metabolic rate is the rate of energy utilized by an organism for basic cellular and organ functions (eg. muscle contract, heart beat, respiration) in fasting and resting state, and at thermoneutrality(10)(Figure 1.1) REE is the largest component of total energy expenditure (TEE) as it accounts for ≈ 65-70 % of TEE, therefore is a primary contributing factor to obesity. In addition, there is an increase in energy expenditure in response to food intake (thermic effect of food) which is heat produced during digestion, absorption, metabolism and storage of macronutrients. The thermic effect of food constitutes ≈ 10% of caloric content of meal ingested. Movement related or physical activity energy expenditure is energy expended by the skeletal muscle for any type of physical movement and is the most variable component of TEE(2). Apart from the three main components of energy expenditure, there is heat production in response to environmental temperature or diet (adaptive thermogenesis) such as cold-induced shivering thermogenesis in skeletal muscle, cold-induced non-shivering thermogenesis in brown fat and diet-induced thermogenesis in

brown fat(11). Lastly, a small proportion is lost in the faeces and urine and a proportion is also used for physiological needs such as growth, pregnancy or lactation(2).

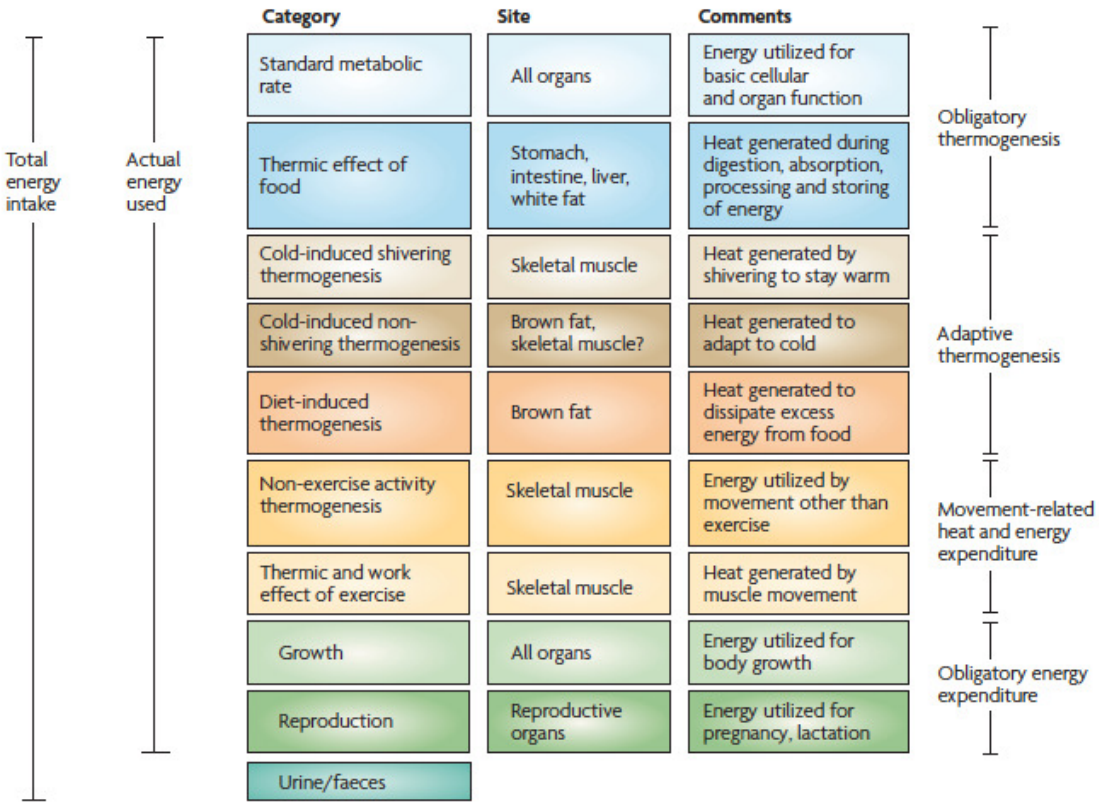


Figure 1.1 Components of total energy expenditure(2)

1.3.2 Low resting energy expenditure is a potential determinant of body weight gain

The hypothesis that low REE is a predictor of weight gain is still controversial. Cross-sectional studies have shown that REE is higher in obese compared to nonobese subjects(12). When REE is normalized for fat-free mass (FFM), the metabolically active compartment, no difference is observed between obese and nonobese subjects(8) As body weight increases, REE rises towards a new equilibrium due to the increase in FFM to limit further body

weight gain, which may play a confounding role in cross-sectional studies(13). Therefore, prospective and longitudinal studies represent the only ways to identify whether or not low REE is associated with body weight gain. Studies in adult nondiabetic Pima Indians have reported the inverse association between baseline REE and body weight gain. Ravussin et al reported that individuals within the lowest tertile of REE had an 8 fold greater risk of gaining 10 kg in body weight over a 4-year follow-up compared with those within the higher tertile of REE(14). The findings were subsequently confirmed in a separate group of Pima Indians, where weight change (-9 to 26 kg) over 4 years of follow up was negatively associated with adjusted REE(15). In Caucasian Italians, Buscemi et al demonstrated that REE was significantly lower in subjects who gained weight ($\geq$ 5kg) than in those who did not (108 ±2.1 vs. 122 ±3.1 kJ/kg-FFM, $P<0.001$)(16). In contrast, low REE was not found to be a predictor of body weight gain in other adult populations. Luke et al observed a positive association between weight change over 5.5 years of follow-up and REE adjusted for sex, age, body size and composition in lean Nigerian, suggesting that increased REE in this population was the result, rather than cause, of weight gain(17). No association between REE and weight change in Nigerian adults was observed(18). It remains to be observed whether the lower metabolic rates that have been observed in African-Americans will relate to subsequent weight gain. These studies may indicate that other factors such energy expenditure due to physical activity, energy intake or the change in energy balance over time and the ability to regulate body energy stores may be more important determinants of obesity rather than REE.

1.3.3 Determinants of resting energy expenditure

Over the past decade, it has been shown that REE is determined by multiple factors including age(19), sex(20), ethnicity(21-23), body composition(24) and body fat distribution(25, 26). FFM remains the principal determinant of REE accounting for 50-70% of the variability in REE, being more metabolically active than fat mass(1, 27). However, it is argued that the close relationship between FFM and REE reported by many studies is artificial and attributed to the large heterogeneity of the subject population(28). Conversely, if the body weight or body composition of the subjects were relatively homogenous, the REE variability linked to body composition would tend to be low(29). An important problem arising from the use of FFM as metabolically active tissue surrogates is that the ratio of REE to FFM is not constant among individuals but varies systematically with body weight(30, 31). Specifically, individuals with smaller body mass and FFM tend to have higher REE: FFM ratios compared to heavier individuals, indicating higher energy production rates per unit of metabolically active tissue. Furthermore, when REE is regressed on FFM, a positive intercept (180-660 kcal/day) exists, implying that a significant component of REE remains even when FFM or body mass is extrapolated to zero(32)

1.3.3.1 High metabolic rate organs and tissues

Studies have demonstrated that a large proportion of inter-individual variation in REE can be explained by heterogeneity in the proportion of FFM as various organ and tissues(33, 34). FFM is a heterogeneous compartment, consisting of organs and tissues with large differences in the rates of energy flux. Compared with the resting metabolic rate of skeletal muscle (14.5 kcal/kg/d), the

metabolic rate of the brain is 18-fold higher (240 kcal/kg/d), of the heart and kidneys is 33-fold higher (440 kcal/kg/d), and of the liver is 15-fold higher (200 kcal/kg/d)(27). Gallagher et al demonstrated that high metabolic rate organs brain, liver, heart, kidneys and gastrointestinal tract account for a disproportionately large fraction of REE relative to their mass (Figure 1.2). They contribute 60–70% of REE in adults, whereas their combined weight is less than 6% of body weight(1). Among the high metabolic rate organs, liver constitutes 2% of body weight but contributes 17% of REE, brain constitutes 2% of body weight but contributes 20% of REE, kidney constitutes 0.5% of body weight but contributes 6% of REE, heart constitutes 0.4% of body mass but contributes 11% and gastrointestinal tract constitutes 2% of body weight but contributes 10% of REE(10). In contrast, skeletal muscle tissue composes 40–50% of total body weight but accounts for only 20–30% of REE. Adipose account for 15 % of body weight and contributes 4% of REE tissue even though they are metabolically less active(1).

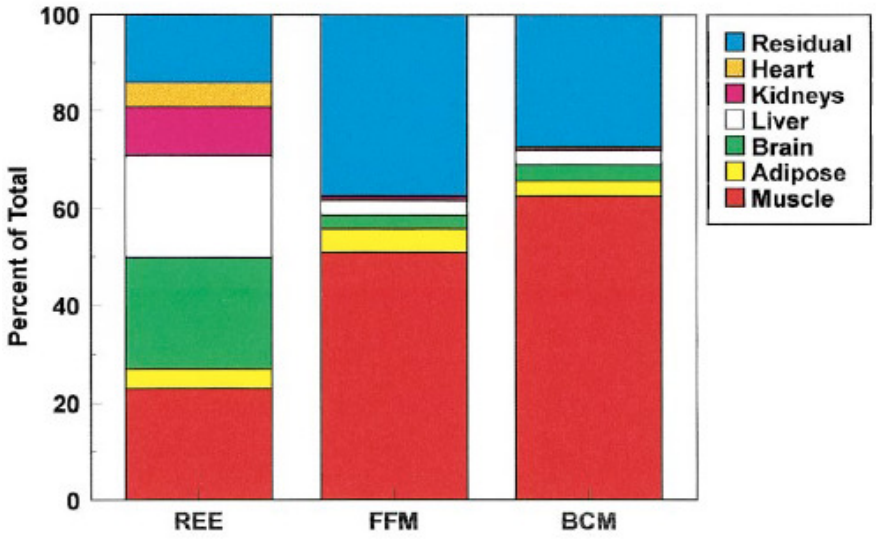


Figure 1.2 Proportional contribution of organs and tissues to resting energy expenditure (REE) and fat-free mass (FFM) (1)

1.3.3.2 Ethnicity

Numerous studies have reported lower levels of REE, adjusted for body size and composition, in African-Americans than Whites (Table 1.1). As a result of these findings, several investigators have suggested that a lower REE may be an important determinant of the higher prevalence of obesity observed in African-Americans than in Whites. Foster et al(35) and Jakicic et al(36) found a lower REE in obese African-American women than in obese white women. Sharp et al reported lower REE in African-American men than White men, in both lean and obese individuals(37). Tershakovec et al also reported that REE is lower in young African-American girls than White girls(38). In contrast, there are studies demonstrating no significant differences in REE between African-Americans and Whites(39). Luke et al(40) also observed similar REE levels between African-Americans and Nigerians, two genetically related populations with dramatically different prevalence of overweight and obesity, casting doubt on the contributing role of REE to ethnic differences in obesity.

Table 1.1 Summary of studies that examined the impact of ethnicity on resting energy expenditure in relation to body weight and body composition. Studies are in chronological order.

Study	Subject characteristics	Measurement of REE	Measurement of body composition	Main finding
Weyer,1990	38 African American and 288 white	Respiratory chamber	Hydrodensitometry	African Americans had lower REE than whites after adjustment for sex, age, and body composition,
Nicklas, 1996	28 African-American and 29 Caucasian women	Indirect calorimetry	DEXA	African American had similar REE to Caucasian women
Jakicic, 1998	41 women (22 African-American and 19 Caucasian)African-American women	Dilution technique	DEXA	REE was lower in African-Americans compared to Caucasians, after adjustment for body weight and lean body mass
Soares, 1998	96 Indians and 81 Caucasian Australians	Indirect calorimetry	Skinfolds	Absolute REE and REE adjusted for body weight were significantly lower in Indians when compared with Australians. However, REE adjusted for FFM, was not significantly different between the two groups.
Luke,1999	89 Nigerian and 181 black adults	Indirect calorimetry	BIA	REE was not different between Nigerian and Black adults
Foster,1999	109 obese females (24 black, 85 white)	Indirect calorimetry	Densitometry	REE, adjusted for body composition, was lower in black than in white subjects
Hunter, 2000	18 African American and 17 white premenopausal women	Room calorimeter	4-compartment model and DEXA	African American women had lower REE than did the white women. Racial differences persisted after adjustment for limb lean tissue but disappeared after adjustment for trunk lean tissue

Abbreviations used: REE (resting energy expenditure), DEXA (dual energy X ray absorptiometry), BIA (bioelectrical impedence analysis), FFM (fat-free mass)

Table 1.1 Summary of studies that examined the impact of ethnicity on resting energy expenditure in relation to body weight and body composition. Studies are in chronological order. (continued)

Study	Subject characteristics	Measurement of REE	Measurement of body composition	Main finding
Sharp, 2001	100 African-American men, 95 white men, 94 African-American women, and 106 white women	Indirect calorimetry	DEXA	REE was higher in white than in African-American individuals, even after adjustment for FFM, fat mass, visceral fat, and age. The difference was the same for men and women and for lean and obese individuals.
Tershakovec, 2002	Obese children and adolescents. 66% were girls and 34% were African American.	Indirect calorimetry	DEXA	Adjustment for trunk lean tissue mass only partially explains the lower REE of obese African American children and adolescents.
Byrne, 2003	18 white and 22 black women	Indirect calorimetry	DEXA	REE adjusted for total LBM and fat mass, was higher in white women
Jones, 2004	22 pairs of age matched African American and white women	Indirect calorimetry	MRI and DEXA	Lower REE in African-American women than in white women was fully explained by differences in the proportions of high metabolic rate organs
Gallagher, 2006	64 women (34 African Americans, 30 whites) and 35 men (8 African Americans, 27 whites)	Indirect calorimetry	MRI and DEXA	Racial differences in REE were reduced by 50% and were no longer significant when the mass of specific high-metabolic rate organs was considered.
Wouters-Adriaens, 2010	10 white men, 10 Asian men, 10 white women and 11 Asian women	Indirect calorimetry	Hydrodensitometry	Absolute REE was lower in Asians than in whites. There was no significant difference in REE between the two races after adjustment for FFM
Javed, 2010	55 women (30 African Americans and 25 whites) and 32 men (8 African Americans and 24 whites)	Indirect calorimetry	MRI and DEXA	Lower REE in African-Americans than Whites was fully explained by trunk high metabolic rate organs.

1.3.3.3 Adipokines

Adipose tissue is an active endocrine organ secreting adipokines such as leptin and adiponectin shown to play important roles in energy balance (41). Administration of recombinant leptin in leptin deficient ob/ob mice induced weight loss by decreasing food intake and increasing resting and physical energy expenditure(42-44). Kubota et al have showed that in mice, adiponectin activates AMP-activated protein kinase (AMPK) by adiponectin/adiponectin receptor (adipo-R1), which is associated with increased phosphorylation of acetyl-CoA carboxylase (ACC) in the hypothalamic ARC, leading to stimulation of food intake and reduction of energy expenditure(45). Several studies have reported positive(39) or negative(46) associations between circulating leptin levels and REE, whereas other studies have no association(47, 48) between leptin and REE. The effect of adiponectin in REE human studies is also controversial. Some studies(49, 50) have shown negative associations between total adiponectin and REE even after adjustment for body composition while others have failed to replicate this result(51, 52). Discrepancies in findings may reflect inconsistencies in accounting for the confounding effect of fat mass on the relationship between leptin, adiponectin and REE.

Recently, novel adipokines such as fibroblast growth factor 21 (FGF21) and apelin have been implicated in the regulation of body mass and whole body energy homeostasis. FGF21 is expressed in liver, white adipose tissue (WAT), skeletal muscle, and pancreas. Administration of FGF21 ameliorated obesity phenotypes in diet induced obese (DIO) and ob/ob mice by increasing oxygen

consumption, core body temperature, even though no decrease in total calorie intake or effect on physical activity were observed(53). Moreover, injection of FGF21 in neonates enhanced the expression of genes involved in thermogenesis within brown fat and increased body temperature(54). According to Holland et al, FGF21 is a potent regulator of adiponectin secretion and critically depends on adiponectin to exert its glycemic and energy metabolism effects. Lee et al recently reported that in humans, mild cold-induced increase in FGF21 was associated with cold induced thermogenesis independent of age, gender, fat mass, and lean mass(55). They went on further to show that FGF21 treatment enhanced brown fat thermogenesis in human adipocytes in a depot-specific manner(56).

Apelin is a novel bioactive peptide discovered from bovine stomach extracts as an endogenous ligand of the G-protein-coupled 7-transmembrane receptor, APJ(57). Active forms of apelin are expressed in many peripheral tissues (heart, lung, kidney, liver, adipose tissue, gastrointestinal tract and endothelium) and different brain regions, particularly the hypothalamus(58, 59). Studies in rodents suggest that apelin has functional roles in regulating energy metabolism. Higuichi et al reported that apelin attenuated body adiposity in normal and diet induced obese mice by increasing energy expenditure mediated by brown adipose tissue uncoupling protein 1 (UCP1)(60). Dray et al demonstrated that apelin decreases glycemia by stimulating glucose uptake in skeletal muscle through an AMP-activated protein kinase (AMPK)-dependent pathway(61). In the study of Yamamoto et al, apelin stimulates energy expenditure by increasing vascular mass, aerobic

type-1 muscle fibre ratio and mitochondrial biogenesis in skeletal muscle(62). Emerging evidence has also shown that MCP-1 may promote chronic low-grade inflammation through pro-inflammatory cytokines, increasing energy expenditure in a regulatory-feedback manner to prevent fat accumulation in obesity(63-67). MCP-1 is a key molecule that mediates infiltration of adipose tissue macrophages (ATM), the dominant source of production of pro-inflammatory cytokines during adipose tissue expansion(68). A study have shown that mice overexpressing MCP1, exhibited an increase in energy expenditure and resistance to diet induced obesity(66)

1.3.3.4 Skeletal muscle

Variability in energy expenditure has been shown to be associated with variability in skeletal muscle metabolism (69, 70). Because of its relatively low resting energy metabolism, skeletal muscle has often been neglected when trying to explain inter-individual differences in REE. Since skeletal muscle accounts for 40-50% of body weight in non-obese subjects, it can account for 20-30% of total resting oxygen uptake (1, 71). Zurlo et al demonstrated that whole body REE normalized for age, sex, FFM and fat mass positively correlated with forearm resting oxygen uptake (69). Astrup et al showed that skeletal muscle is the principal site of thermogenesis, accounting for 40% of adrenaline-induced thermogenesis(70, 72). Several factors might influence the variation in muscle energy expenditure, including thyroid hormone, muscle tone, sympathetic innervation, catecholamine levels, and muscle fiber type composition Studies have also reported the association between REE and maximal activities of enzymes involved in energy-generating pathways (ie glycolysis, β-oxidation, tricarboxylic acid cycle, electron transport chain) such

as phosphofructokinase (PFK)(73), citrate synthase, cytochrome-c oxidase (COX) and 3-hydroxyacyl-CoA dehydrogenase (HADHA)(74).

1.3.3.4.1 Mitochondrial energy metabolism

The oxidation of fatty acids and pyruvate takes place in mitochondria where energy is converted into ATP for use in cellular processes (Figure 1.3). In the tricarboxylic acid (TCA) cycle, acetyl-CoA from fatty acid oxidation and glycolysis is oxidized to reduced coenzymes, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2), which deliver their electrons to the electron transport chain (ETC) located on the inner mitochondrial membrane. As electrons move down through the complexes of the ETC, energy is used by complexes I, III and IV to pump protons outside of the mitochondrial inner membrane, creating an electrochemical potential gradient. During oxidative phosphorylation, protons are driven back into the matrix through the F_1F_o-ATP synthase and the potential energy in the proton motive force is used to drive conversion of ADP to ATP (11). If ADP is unavailable, protons are unable to enter through ATP synthase, causing the elevated electrochemical proton gradient to put backpressure on proton pumps in the ETC, inhibiting substrate oxidation and mitochondrial respiration (75). Hence, energy expenditure can be increased by increasing utilization of ATP, thus increasing availability of ADP or "uncoupling" of the tight relationship between fuel oxidation and ATP production, allowing fuels to be oxidized in the absence of ADP. Classically, two major biochemical systems are believed to contribute to energy expenditure: (1) futile cycles which increase utilization of ATP such as protein turnover, ion cycling across plasma membranes (Ca^{2+} ATPase) and

triglyceride-fatty acid substrate cycling and (2) mitochondrial proton leak(76).

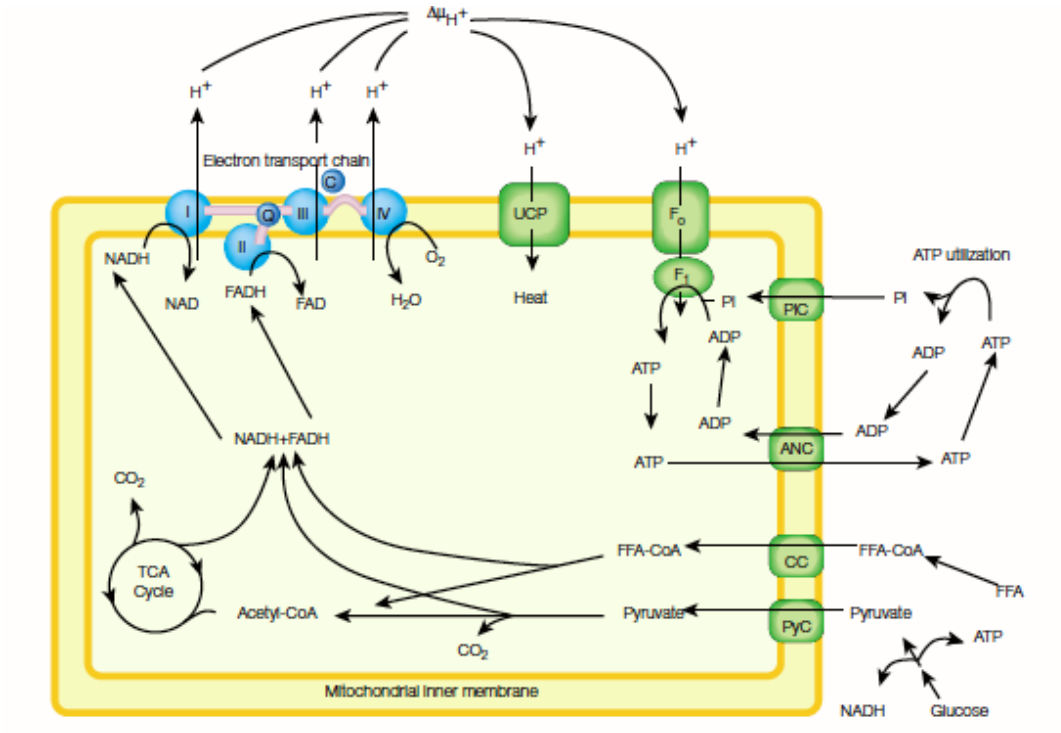


Figure 1.3 Mitochondrial energy metabolism

1.3.3.4.2 Contribution of ATP consuming reactions to REE

Several ATP consuming reactions for energy dissipation are protein turnover, futile calcium cycling and triglyceride-fatty acid substrate cycling. Protein turnover, which is the degradation of proteins into amino acids and resynthesis of new proteins has been estimated to account for 15-20% of REE in humans (77). Most tissues exhibit protein turnover, especially the skeletal muscle tissue (25% of total protein turnover), skin (18%), liver (24%), and small intestine (15%)(77). Welle et al demonstrated in healthy young subjects that REE is highly correlated with leucine flux, an index of proteolysis and non-oxidized portion of leucine flux, an index of protein synthesis(78). Conditions such as hyperthyroidism, diabetes, leukemia and overfeeding produce an

increase in both REE and protein turnover, whereas under-nutrition reduces both REE and protein turnover. REE and protein turnover are also increased in patients with severe chronic obstructive pulmonary disease(79). In both short term and long term studies, high protein diets have shown to increase energy expenditure and improve body weight control(80, 81). After a fast in humans, whole-body nitrogen turnover and the thermic response to protein diet feeding were found to be significantly greater when compared with a high-carbohydrate meal(82).

Previous work has shown that Ca^{2+} adenosinetriphosphatases (ATPases) accounted for 20% of resting metabolic rate in adult rats(83, 84). The thermogenic potential of futile Ca^{2+} cycling is evident from the pathological syndrome of malignant hyperthermia, a dominant genetic disorder of humans due to a mutation in the skeletal muscle ryanodine receptor (Ryr), the calcium release channel of the sarcoplasmic reticulum. Abnormal Ca^{2+} release, triggered by anaesthesia or stress, causes intense thermogenesis, which leads to hyperthermia(85). Cold exposure in uncoupling protein 1(UCP1) deficient mice showed an increase in Serca2a (sarcoplasmic reticulum Ca^{2+} ATPase) expression, which enabled a calcium cycling induced rise in energy expenditure(86). In hyperthyroid patients and healthy controls, Ca^{2+} ATPase content in skeletal muscle correlated with REE(87).

In triglyceride-fatty acid cycling, fatty acids are released during lipolysis and subsequently re-esterified rather than oxidized(88). It was reported in animal models that substrate cycling between de novo lipogenesis and lipid oxidation

is stimulated by leptin, causing an increase in energy expenditure(89, 90) It has also been shown in humans that mild-cold exposure increased fatty acid cycling together with energy expenditure (91). Recently, Mainieri and colleagues reported a role for skeletal muscle stearoyl-CoA desaturase 1(SCD1) in thermogenesis by desaturating the products of de novo lipogenesis and diverting them away from mitochondrial oxidation, hence inhibiting substrate cycling between de novo lipogenesis and lipid oxidation, thereby leading to a state of suppressed thermogenesis(92). The triglyceride/fatty-acid cycling is also involved in the thermogenesis associated with burn injuries(93), cancer cachexia(94), and after exercise(95)

1.3.3.4.3 Contribution of mitochondrial proton leak to REE

According to Rolfe and colleagues, mitochondrial proton leak accounts for 50% of oxygen consumption rate of perfused, resting skeletal muscle and 25% of oxygen consumption rate of isolated resting hepatocytes in rat(10). In vivo, it has been calculated that mitochondrial proton leak in skeletal muscle and liver alone could account for about 20-25 % of rat REE (96). In humans, mitochondrial proton leak dependent respiration in skeletal muscle was positively associated with cold induced adaptive thermogenesis(97). Obese subjects demonstrated decreased uncoupled respiration per mitochondrial volume compared to lean subjects (98). Diet-responsive subjects who experienced 43% greater weight loss than diet-resistant subjects were shown to have greater skeletal muscle mitochondrial proton leak dependent respiration, suggesting that differences in metabolic efficiency explain some of the variability in weight loss success(99). Hence skeletal muscle mitochondrial proton leak may be an important determinant of energy

expenditure in humans. It is postulated that protons return to the matrix through alternative leak pathways independent of F1Fo-ATP synthase. This proton leak reduces the proton gradient driving ATP formation and uncouples respiration from oxidative phosphorylation, releasing stored energy in the form of heat(100). Adenine nucleotide translocase (ANT) and uncoupling proteins (UCPs) localized on the inner mitochondrial membrane may be involved in proton conductance pathways of mitochondria, hence are likely candidates to underlie the physiological variability in REE in humans. UCP1, UCP2 and UCP3 cause nucleotide-sensitive proton leak when they are activated by fatty acids or alkenals derived from the peroxidation of membrane phospholipids(101). The primary function of ANT is to catalyze ADP/ATP exchange across the mitochondrial inner membrane. Under respiring conditions, ATP^{4-} generated by oxidative phosphorylation is exported to the cytosol for use in cellular activities while ADP^{3-} is imported into the mitochondrial matrix for ATP synthesis. ANT is also thought to have an intrinsic property of mediating proton leakage, to be a regulatory component of the mitochondrial permeability transition pore and to be involved in mitochondria-mediated apoptosis. ANT causes carboxyatractylate (CAT) sensitive proton leak when activated by fatty acids, AMP or alkenal(102). Brand and colleagues demonstrated that ANT accounts for 1/2 to 2/3 of the basal proton conductance independent of its fatty-acid-dependent proton-leak functions (103). There are four different ANT isoforms in humans that have distinct tissue specific expression patterns. ANT1 is predominantly expressed in the mitochondria of heart and skeletal muscle tissue(104), ANT2 is sparse or absent in differentiated tissues, but more abundant in certain

proliferating tissues, ANT3 is ubiquitously expressed and ANT4 is specifically expressed in the testis(105).

1.3.3.4.4 Uncoupling proteins

Uncoupling protein 1 (UCP1) is a mediator of proton leak in brown adipose tissue, which is responsible for nonshivering thermogenesis in newborn humans and in small mammals. UCP1 ablated mice are more susceptible to cold temperatures and to obesity when housed at thermoneutrality (106, 107) In humans, the relevance of UCP1 in adult humans has been controversial as UCP1 is exclusively expressed in BAT. Histological evidence has indicated that BAT is present, albeit in small amounts, in adults throughout life, however attempts to find functional BAT or utilize its thermogenic capacity for weight loss have been largely unsuccessful(108-111). Recently, homologues of UCP1 have been found that are abundant in other tissues than BAT. UCP2 and UCP3, which have an approximate 59% sequence homology to UCP1, have been shown to have uncoupling activity (112-114). UCP2 is widely expressed in skeletal muscle, fat, heart, placenta, lung, liver, kidneys and pancreas while UCP3 is predominantly expressed in skeletal muscle and brown adipose tissue(112, 113, 115). UCP3 gene knockout mice exhibit lower state 4 respiration(116). Walder and colleagues reported an association between polymorphisms in UCP2 and sleeping metabolic rate (SMR) and TEE In Pima Indians(117). Bouchard et al also demonstrated significant associations between UCP2 gene polymorphism and REE in Quebec population(118). Schrauwen further reported that in Pima Indians, UCP3 mRNA expression was positively correlated with REE adjusted for fat-free mass and fat mass and negatively correlated with BMI(119). Recently, Kimm

et al identified a significant association between REE and C allele in the UCP3 exon 5 variant in African American women, which suggested that the UCP3 exon 5 variant may account for lower REE in African American compared to Caucasian women(120).

However, unlike UCP1, the role of UCP2 and UCP3 in regulating proton leak and energy expenditure is a subject of controversy. There are reports that the expression of UCP2 and UCP3 mRNA increases with starvation, a state known to be associated with decreased energy expenditure(121, 122). Elevation of UCP3 by high fat diet did not change skeletal muscle mitochondrial efficiency in humans (measured as the rate of PCr resynthesis after exercise)(123). Animal studies have also shown alternative roles of UCP2 and UCP3 in fatty acid oxidation to prevent lipid induced oxidative mitochondrial damage and the reduction of proton gradient to reduce production of reactive oxygen species (ROS)(112, 113, 116, 124-126). In human studies, many failed to find significant associations between genetic polymorphisms and mRNA of UCP2, UCP3 and REE(110, 127-129). Hence the physiological roles of human UCP2 and UCP3 in mediating energy expenditure are still under debate.

1.4 Metabolic flexibility

Metabolic flexibility (MF) is defined as the capacity for the organism to match fuel oxidation to fuel availability. The inability to modify fuel oxidation in response to changes in nutrient availability has been implicated in the pathogenesis of obesity and insulin resistance. Lean healthy individuals use lipids as a main source of fuel during fasting conditions and they readily

switch to carbohydrate (CHO) oxidation under insulin stimulation. Conversely, the inflexible muscle of obese insulin resistant or type 2 diabetic (T2D) individual is characterized by lower fasting rates of lipid oxidation and impaired ability to suppress lipid oxidation and increase CHO oxidation in response to insulin(130). The impaired ability to augment lipid oxidation during fasting conditions is a key mechanism, leading to accretion of lipid metabolites such as triglyceride, ceramides, diacylglycerol (DAG) and long chain fatty acyl-CoA in the skeletal muscle, which impair insulin signaling and contribute to insulin resistance(131).

Thus far in human studies, MF has been measured as the increase in respiratory quotient (RQ) from fasting to glucose/insulin-stimulated conditions during a euglycemic hyperinsulinemic clamp (EHC) or mixed meal challenge (Table 1.2). Many studies have reported that obese insulin resistant(132) and T2D subjects(133) exhibit elevated fasting RQ and blunted increase in RQ during a hyperinsulinemic clamp. Studies are still lacking to identify mechanisms linking MF during a clamp and insulin resistance. Insulin-stimulated glucose disposal rate is reported to be the main determinant of RQ change during a clamp, explaining 50% of its variance. This metabolic inflexibility is mostly the consequence of impaired cellular glucose uptake rather than defective cellular glucose oxidation(134). Furthermore, after controlling for glucose disposal rate, no improvement in MF is observed after weight loss in T2D subjects(134, 135).

Studies that have evaluated MF using mixed meal challenge have yielded

conflicting results. Corpeleijn reported that postprandial increase in forearm RQ was blunted in impaired glucose tolerant compared to control subjects after a high fat meal (136). In contrast, Helibronn et al observed that in response to a high CHO meal, individuals with a family history of T2D had a similar increase in RQ compared with control subjects(137). Studies also reported no difference in the change in 24-h RQ between lean and obese subjects after 3 days of overfeeding with a high CHO diet, suggesting similar MF between groups(138). In a study by Ukrocopva et al, subjects with or without a family history of T2D had a similar decrease in 24-h RQ after 3 days of high-fat diet(139).

Although the effects of BMI and diabetes status on MF have been observed, contribution of ethnicity to MF has been less explored. To our knowledge, only two studies have reported the significant contribution of ethnicity to MF. Berk et al observed that obese Caucasian women had significantly higher fat oxidation and lower CHO oxidation during the high fat vs. low fat diet, whereas no significant differences in substrate oxidation were observed in African Americans. The African-American women also failed to suppress fat oxidation during pancreatic euglycemic clamp or increase fat oxidation in response to epinephrine infusion compared to Caucasian women(140). Impaired substrate switching in African-Americans may contribute to their greater prevalence of obesity and insulin resistance. In contrast, Stull et al reported greater substrate switching in African-Americans during a hyperinsulinemic euglycemic clamp compared to Caucasians, even after adjustment for insulin sensitivity(141).

Table 1.2 Summary of studies that examined metabolic flexibility during euglycemic hyperinsulinemic clamp (EHC) or mixed meal tolerance test (MMTT)

Study	Subjects	Assessment of MF	Main outcome
Kelley, 1990	15 diabetic subjects was matched to 15 nondiabetic subjects	EHC	Insulin-stimulated leg glucose uptake in the diabetics did not differ from controls after matching for muscle glucose uptake
Kelley, 1990	Sixteen lean and 40 obese	EHC	During fasting conditions, obese subjects had an elevated leg RQ. During insulin infusions, fat oxidation by leg tissues was suppressed in lean but not obese subjects
Kelley, 1994	11 non-insulin-dependent diabetes mellitus (NIDDM) and 9 nondiabetic subjects	MMTT	Postabsorptive leg RQ was increased in NIDDM. During 6 h after ingestion of a mixed meal, arterial free-fatty acid remained greater in NIDDM subjects
Goodpaster, 2003	9 men and 16 women completed 16 weeks of moderate-intensity physical activity combined with caloric reduction.	Fasting	Rates of fat oxidation following an overnight fast increased
Blaak, 2006	701 obese and 113 lean	MMTT (95% fat)	Postprandial fat oxidation decreased with increasing BMI. Fasting fat oxidation increased with increasing BMI.
Ukropcova, 2007	16 men with and 34 men without a family history of diabetes	3-day HFD	Subjects with a family history of diabetes had a significantly higher sleep RQ on the 3rd day of a HFD
Galgani, 2008	59 type 2 diabetic and 42 nondiabetic individuals matched for obesity, sex, and race	EHC	Impaired metabolic flexibility to glucose observed in type 2 diabetic versus nondiabetic subjects was no longer observed after adjusting for glucose disposal rate
Corpeleijn, 2008	Thirteen obese men with IGT and nine obese men with normal glucose tolerance (NGT), matched for age and BMI,	MMTT (33% carbohydrates, 61% fat, 6% protein.)	The postprandial increase in RQ was blunted in IGT compared to NGT, but improved after weight loss

Study	Subjects	Assessment of MF	Main outcome
Stull, 2009	168 human subjects of different races (55 African Americans, 113 Caucasians)	EHC	MF was higher in African Americans vs Caucasians
Huffman, 2011	46 men and 25 women randomized into control group (healthy weight maintenance diet) and caloric restriction(CR) group	MMTT	Larger differences in fasting-to-postprandial concentrations of medium and long chain acylcarnitines (byproducts of fatty acid oxidation) in the CR relative to control group
Weyer, 2011	14 male subjects(7 Caucasians and 7 Pima Indians)	48 h of mixed diet overfeeding and fasting	Changes in 24-RQ in response to overfeeding and fasting were not different between Caucasians and Pima Indians

1.4.1 Role of mitochondrial function in metabolic flexibility

Studies have indicated that mitochondrial abnormalities may be a primary cause of MF, however the causal link between the two remains to be established. Ukropcova et al reported that greater in vitro adaptability (increase in fat oxidation in the presence of high palmitate concentration) and lower in vitro suppressibility (glucose suppression of fat oxidation) correlated with higher metabolic flexibility and insulin sensitivity in healthy young males, thereby hypothesizing that metabolic switching is an intrinsic property of skeletal muscle(142). Myotubes established from patients with T2D exhibit reduced insulin-stimulated glucose uptake, oxidation, and glycogen synthesis, whereas palmitate exposure impaired insulin-stimulated glucose oxidation and insulin stimulated citrate synthase activity in control myotubes(143). Muscle mitochondrial content was higher in metabolically flexible subjects with high fat oxidation after a high fat diet (HFD) and contributed 49% of the variance, further supporting that metabolic switching from carbohydrate to fat oxidation is an intrinsic characteristic of skeletal muscles(139). Similarly, Chomentowski reported that intermyofibrillar mitochondrial content was significantly correlated with fasting RQ and MF(144). In response to high fat meal, subjects with a family history of T2DM had an impaired ability to increase fatty acid oxidation, which was associated with impaired activation of genes involved in lipid metabolism such as peroxisome proliferator-activated receptor coactivator-1(PGC1A) and fatty acid translocase (FAT/CD36)(137). Boyle and colleagues demonstrated that myotubes from lean subjects has increased mitochondrial respiration (state 3) in the presence of palmitoyl carnitine compared to obese subjects, indicating that skeletal muscle of obese

individuals inherently lacks metabolic inflexibility in response to lipid exposure(145). In contrast, some studies suggest that MF may not be a phenotype intrinsic to the skeletal muscle and a contributing factor to insulin resistance and T2D. Gaster and colleagues reported that metabolic inflexibility in obese and diabetic patients is based on the inability to vary extracellular fatty acid concentrations during insulin stimulation(146). Similarly, Weijer reported that reduced mitochondrial function does not negatively impact the ability of skeletal muscle to switch substrates during insulin stimulation, rather it is primarily responsible for basal substrate oxidation. (147).

1.5 Fat mass and obesity associated gene (FTO): first GWAS identified obesity gene

Individuals respond differently to the "obesigenic" environment changes in diet and lifestyle and it is increasingly clear that variation in response has an important genetic element. Monozygotic and dizygotic twin studies have shown greater interpair than intrapair variation in weight loss, indicating that adiposity is highly heritable with the estimated genetic contribution of 40–70% of the variance in weight gain(148, 149). Recognition of an important genetic influence on obesity has led to a search for causal genes which might help us fight this emerging epidemic. Genome wide association studies (GWAS) have identified at least 75 obesity susceptibility loci, including leptin (LEP), pro-opiomelanocortin (POMC), melanocortin 4 receptor (MC4R) and FTO (fat mass and obesity associated)(150). In the first GWAS study, single nucleotide polymorphisms (SNPs) in the first intron of FTO gene were found to be strongly linked with both BMI and T2D (151). However, the association

with T2D was abolished after adjustment for BMI, suggesting that the SNP predisposes to T2D through an effect on body weight. Of all GWAS-identified obesity susceptibility loci, the FTO gene has the most robust association with human obesity and greatest effect size(152). The minor risk allele increases BMI by 0.39 kg/m^2 and risk of obesity by 1.20-folds(153). This genetic association was demonstrated across all age groups and in more than 22 different ethnic populations including Chinese, Malay and Asian-Indian populations in Singapore(150, 154-156).

A recent study showed that obesity associated FTO introns are functionally connected with the promoters of IRX3 rather than promoters of FTO through long range interactions. FTO SNP is associated with increased IRX3 expression, and not with FTO expression in human brain. They further demonstrated that IRX3-knockout mice weigh about 30% less compared to wild types, primarily through loss of fat mass, increase in basal metabolic rate and browning of WAT(157). Although these findings with IRX3 are intriguing, they do not provide conclusive evidence against a contribution by FTO. Other lines of evidence that support a role for FTO in obesity comes from several animal studies where inactivation of FTO protein is found to protect from diet-induced obesity(158). In particular, FTO-/- and FTO^{I367F} (mouse with a dominant missense I367F mutation in the FTO gene) mice were found to have significantly reduced adipose tissue, apparently due to an increase in energy expenditure and a decrease in lipogenesis(158, 159). All these results suggest that FTO is potentially the bonafide target of obesity.

1.6 Rationale of Study

This study is novel in identifying the most accurate equation appropriate for predicting energy needs in the Singaporean Chinese population, which is important in establishing adequate dietary intake goals for effective weight management of overweight or obese individuals in obesity clinics, nutritional management of hospitalized patients to avoid the adverse effects of overfeeding and underfeeding and estimation of the energy requirements of a population. This area has considerable public health potential.

In addition, this study is novel in examining differences in resting energy expenditure (REE) and fuel utilization among Chinese, Malays and Asian-Indians in Singapore, which may be particularly important given that these, and related, populations, represent up to 2/3 of the world's population. Most studies looking at ethnic variability in REE and fuel utilization were completed in North American populations and specifically focused on African American/Caucasian differences. An understanding of ethnic differences in these metabolic risk factors for obesity will facilitate the development of targeted and more efficacious interventions for the overweight individual based on ethnicity.

Current approved clinical approaches for treatment of obesity such as energy restricted diets, increased physical activity and pharmacological treatments have limited impact. Current anti-obesity drugs aim to reduce food intake by either curbing appetite or suppressing the craving for food. Due to their central mode of action, they suffer from severe psychiatric and/or cardiovascular side

effects, many of which are withdrawn from the market as a result. Bariatric surgery, arguably the only effective treatment we have for obesity, is associated with serious risks and complications. While there is little doubt about the benefits of exercise and healthy diets in improving overall health, it is evident that additional approaches must be explored to improve the long-term effectiveness of interventions. Losing weight by caloric restriction is not successful in the long term as weight loss causes a reduction in REE, which could be a risk factor for body weight gain, making further weight loss even more difficult(160). Structured exercise programs designed to increase TEE have only a low efficiency and have generated mixed results. The limited potency for physical activity to increase TEE may be due to the fact that the caloric cost per time unit of common exercise types are typically low compared to caloric intake. Hence targeting cellular energy expenditure (bioenergetics) via non-exercise means may be an attractive alternative approach. The molecular mechanisms underlying variability in energy metabolism and fuel utilization in human skeletal muscle are largely unknown. Proper characterization of these molecular mechanisms in human skeletal muscle is particularly important in the discovery and validation of therapeutic targets that might be useful and promising for the pharmacotherapy of obesity and its associated complications.

In particular, FTO is an attractive therapeutic target for obesity and related metabolic diseases because it not only shows robust link with obesity in humans, the protective FTO allele is also not known to be associated with any adverse phenotypes. This potentially translates to a safer and more efficacious

approach to obesity treatment compared to conventional targets. Although there is compelling evidence that an increase in FTO expression underlies the obesity phenotype, the exact mechanism by which FTO regulates weight and energy homeostasis remains to be elucidated. It is, therefore, of clinical interest to understand the physiological functions and biological significance of FTO and explore its epigenetic link to obesity. Our research could pave the way for novel anti-obesity drugs that target the genetic causes of obesity.

Overall, our study findings have potential public health, diagnostic, pharmaceutical and translational opportunities for targeted intervention and prevention of obesity.

1.7 Aims of Study

This study aims to explore the impact of ethnicity on resting energy expenditure (REE) and fuel utilization in Chinese, Asian-Indian and Malay men and characterize the underlying mechanisms by which FTO (fat mass and obesity associated gene) regulates body weight and energy homeostasis. Specific aims of this study are to:

1. Evaluate the validity of Food and Agriculture Organization/World Health Organization/United Nations University (FAO/WHO/UNU) equation and other commonly used prediction equations, Harris Benedict, Mifflin et al and Owen et al for REE in a sample of healthy Singaporean Chinese men.

2. Determine whether ethnicity is associated with REE and the role of fat free mass (FFM), fat mass and mass/volume of high metabolic rate organs (represented by brain volume and trunk FFM) to these ethnic differences in REE. We further sought to examine whether inter-individual variation in circulating concentrations of adipokines help to account for inter-individual variation in REE once the effects of FFM and fat mass had been accounted for.

3. Determine whether metabolic flexibility is associated with ethnicity, obesity and insulin resistance in Chinese, Asian-Indian and Malay men. We further sought to understand any associations observed could be explained by plasma metabolic intermediates (acylcarnitines,

organic acids and amino acids) using metabolic profiling, and plasma levels of adipokines

4. Determine whether potential inter-individual differences in skeletal muscle characteristics can explain variability in REE and MF and also the impact of ethnicity on molecular factors affecting skeletal muscle energy metabolism

5. Characterize the function of FTO in skeletal muscle energy metabolism and substrate utilization and examine how the N6-methyladenosine (m^6A) demethylation activity of FTO contributes to its metabolic effects and obesity using two approaches a) genetic approach by gene silencing and b) chemical probe approach using a novel pharmacologic inhibitor (CA) of FTO m^6A demethlyase activity.

Chapter 2 : Materials and Methods

2.1 Study Design

The Singapore Adult Metabolism (SAM) study is a cross sectional study designed to investigate the contribution of ethnic, genomic and developmental factors to the variation in phenotype observed in adults with obesity and metabolic syndrome. This study involves detailed phenotyping of adults that combines a variety of approaches including metabolic imaging, experimental physiology in humans and molecular biology using primary human myotubes as the in vitro experimental model. The study was granted ethical approval by the Domain Specific Review Board of the National Healthcare Group and written informed consent was obtained from each subject. We recruited 264 healthy adult males, comprising 101 (38.3%) Chinese, 82 (30.7%) Malays and 81 (31.1%) Asian-Indians. For screening, study subjects were required to fast overnight (10–12 hours). Following informed consent, baseline anthropometric measurements, complete medical history, physical examination and blood pressure (in mmHg) were recorded. Following screening, eligible subjects will enter the study. Subjects were recruited by advertisement.

2.2 Eligibility

2.2.1 Inclusion criteria

Participants must meet all of the following to criteria to participate in this study

- Males aged 21-40 years. We have chosen to study men in order to avoid potential gender bias confounded by the menstrual cycle on metabolic parameters. However, it should be noted that this experiment reflects an initial proof of concept and we will later extend our studies to include pre menopausal women based on the findings of this initial study. The selection of a relatively young study population avoids potential confounding by other co-morbidities and drug treatment.
- Body mass index (BMI) 18.5-30.0 kg/m^2 .We defined lean individuals as BMI 18.5-22.9 kg/m^2, overweight as BMI 23.0-27.4 kg/m^2, and obese as BMI $\geq$ 27.5 kg/m^2. This was in line with the WHO expert consultation in 2002 that in Asian populations, BMI 18.5-23.0 kg/m^2 represents increasing but acceptable risk, BMI above 23.0 kg/m^2 represents increased risk, and BMI above 27.5 kg/m^2 represents high risk for cardiometabolic disease
- Fasting plasma glucose below the threshold for diagnosing T2DM (<7.0 mmol/L)
- Normotensive, defined as blood pressure <140/90 mmHg
- Sedentary lifestyle defined as exercise <1 time/week of 30 minutes duration

2.2.2 Exclusion criteria

All subjects meeting any of the exclusion criteria at baseline will be excluded from participation.

- Treatment for diabetes mellitus, hypertension and dyslipidemia in view of the nature of the study to examine the degree of physiological and metabolic derangements among an overtly healthy group, and being on

treatment would interfere with the interpretation of metabolic parameters. While we exclude participants who were already on treatment for hypertension, dyslipidemia or diabetes mellitus, those having the features suggesting metabolic syndrome (as defined by locally accepted guidelines, e.g. NCEP/MOH/WHO/AHA/ACC) but who were as yet untreated were also included as it was expected that a significant number of overweight and obese participants with the metabolic syndrome may be asymptomatic and thus appear overtly healthy

- Use of medications that will likely affect energy expenditure (eg. sibutramine) and insulin resistance (eg. glucocorticoids, ACE-inhibitors, protease inhibitors, metformin). Similarly, participants on an investigational drug in the past 6 months will be excluded as it would not be predictable if the agent has any effect on metabolic parameters.
- Recent changes in body weight of >5% over the past 6 months or who were actively attempting to lose weight through dieting, bariatric surgery or anti obesity drugs were excluded in view of the need to recruit participants with stable weight of a given narrow distribution of BMI
- Bleeding disorders which would preclude biopsies
- Known allergy to insulin or local anaesthesia
- Known allergy to milk or milk products (eg. Ensure)
- Presence of cardiac pacemaker or metallic clips, staples or stents in any part of the body which would preclude MRI/MRS

- Any serious illness requiring hospitalization or surgery in the past 6 months
- Use of any prescription medication that cannot be safely discontinued within 14 days prior to study entry

2.3 Assessment of Anthropometry and Body Composition

Demographic data, medical and drug history, and data on lifestyle factors were collected using interviewer-administered questionnaires. Body weight was measured in light clothing to the nearest 0.1 kg using an electronic scale (SECA, Vogel & Halke, Germany). Height was measured without shoes to the nearest 0.1cm using a wall-mounted stadiometer. BMI was calculated as weight divided by height squared (kg/m^2). Whole body and regional (trunk, arms and legs) lean body mass (LBM), fat mass and bone mineral content (BMC) were measured by dual-energy x-ray absorptiometry (DEXA; Hologic Discovery Wi). BMC was used in the calculation of whole body fat percentage by the 4-compartment model(161). Fat-free mass (FFM) was calculated as the sum of LBM and BMC. Limb FFM was calculated by summing both arm and leg FFM. Limb FFM was used as an index of total skeletal muscle mass as suggested by Heymsfield et al (162) while trunk FFM was used as an index of visceral organ mass in the chest and abdomen (liver, kidneys, spleen and heart, gastrointestinal tract)(22). The coefficient of variation values for repeated measurements were 0.9%, 1.3%, and 2.2% for LBM, BMC and fat mass respectively.

2.4 Assessment of energy expenditure and respiratory quotient (RQ)

On the test day, subjects reported to the National University Hospital at 0830 h. They were instructed to transport themselves to the hospital in a vehicle to avoid undue exertion. They were required to undergo a 10 h overnight fast and refrain from intensive physical activity for 24 h prior to measurement. They were also advised to abstain from coffee and other nicotine containing food or beverage, heavy meals, and alcohol in the evening prior to measurement. Subjects were then allowed to lie supine quietly and relaxed for 15 min before measurement commenced. Measurements of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were performed by open-circuit indirect calorimetry using a ventilated hood system (Quark CPET, COSMED, Italy) for 1-hour in the fasting condition and continuously measured for a further 3-hours following a mixed-meal tolerance test (MMTT). The MMTT was a standardized liquid meal (Ensure Plus®, Abbott Nutrition, Abbott Laboratories, Columbus, OH) with a total calorie content of 600kcal which comprised 55% from carbohydrates, 30% from fat (10% saturated fats, 10% polyunsaturated fats, 10% mono-unsaturated fat), and 15% from protein). A transparent Perspex ventilated hood was placed over the subject's head, through which outside air was drawn by a pump. The flow rate (20—40 L/min) was measured and adjusted to keep the difference in carbon dioxide readings between inspired and expired air within the range of 0.8—1.2%. A small sample of air leaving the hood was analyzed for oxygen (O_2) and carbon dioxide (CO_2) by a paramagnetic analyzer and infrared analyzer respectively. Calibration of flow and gas analyzers were done before each measurement according to the manufacturer's instructions. Flow calibration was performed

by a 3-litre calibration syringe and gas analyzers were calibrated with dried standard gas mixture (16.01% O_2, 4.98% CO_2) and dried atmospheric air (20.93% O_2, 0.03% CO_2) The validity of the ventilated hood system was further tested by ethanol combustion tests conducted biweekly. The mean O_2 recovery was 98.8 ±1.9% (95% confidence interval 98.7 and 99.0) and the mean CO_2 recovery was 98.3 ± 1.6% (95% confidence interval 98.2 and 98.4) (unpublished results). Correction factors were applied when necessary. All measurements were carried out in a quiet room with an ambient temperature of 23—25 ◦C, barometric pressure of 750—770 mmHg and constant humidity of 60%. During the measurements, subjects were lying in a semi-supine position, quiet, motionless and were kept awake by showing them non-stressing movies, which were vetted for violent or sexual content that may excite them. To eliminate effects of subject habituation to the testing procedure, the respiratory measurements during the first 5 min were discarded. Only steady state periods of measurement of at least 30 min were used. REE was calculated from VO_2 and VCO_2 by the Weir formula(163). RQ was calculated from VO_2 and VCO_2 as follows: $RQ=VCO_2/VO_2$.

2.5 Magnetic resonance spectroscopy (MRS) of liver and skeletal muscle adipose tissue

Fat content in the liver and skeletal muscle were determined using 1H MRS using a 3 Tesla MR scanner (Tim Trio, Siemens)(164). The liver spectra were obtained from a 2Å~2Å~2cm^3 voxel from two locations (right and left lobes) using a PRESS sequence (TE/TR = 30/2000ms) and a Siemens body matrix coil. The acquired spectra were fitted using the linear combination of model spectra (LCModel). The liver fat was determined from the concentration of

methyl and methylene groups of lipids and unsuppressed water signal. The fat concentration obtained was corrected for T2 losses and was also verified by region-of-interest analysis with Dixon imaging. For skeletal muscle spectroscopy, the subjects' right leg was positioned in a leg holder and the foot was aligned to eliminate residual dipolar interactions. The muscle spectrum was obtained from a 2Å~2Å~2cm3voxel within the soleus muscle using a PRESS sequence (TE/TR = 30/2000ms) and a Siemens Tx/Rx 15-Channel Knee Coil. The amount of IMCL was calculated and expressed as a ratio to Creatine (Cr), i.e. IMCL/Cr.

2.6 Magnetic resonance imaging of abdomen

Abdominal fat images were acquired using two 2-point DIXON sequences (TR=5.28ms, TE1=2.45ms, TE2=3.68ms, FA=9deg, slice thickness=3mm) during breath-holds of 18–20 seconds. Fat volume in each abdominal fat compartment was obtained from 80 axial slices covering the L1 to L5 lumbar vertebrae. A fully automatic graph theoretic segmentation algorithm extracted and estimated the subcutaneous (SAT) and visceral (VAT) adipose tissue volumes. The segmentation algorithm was a two-step process. First, the fat tissues were separated from the non-fat tissues by thresholding. The extracted fat tissues were then classified into SAT and VAT using a graph cut technique which has been validated on the skull stripping problem(165). The segmented image volumes of each of the fat depots were quantified by adding all the voxels of all the slices and multiplying by the image resolution. This method provides valid estimates of fat volume compared to manual segmentation with Dice similarity index ranging from 0.7-0.89 (where 0 indicates no overlap between the 2 methods and 1 indicates perfect overlap).

2.7 Magnetic resonance imaging of brain

High-resolution images of the brain were acquired using a T1- weighted MP–RAGE sequence. There were 192 contiguous sagittal slices with the following scanning parameters: TR=2530 ms, TI=1200 ms, flip angle=7˚, FOV 256mm×256 mm, 256×256 matrix, isotropic voxel dimensions of 1.0mm, 6min 3s acquisition time. Automated measurements of brain volumes were performed using FreeSurfer 4.5.0 (http://surfer.nmr.mgh.harvard.edu/). Total brain volume was calculated as sum of total cerebral gray matter, cerebral white matter, brain stem and cerebellum(149) Estimated total intracranial volume (eTIV) calculated using a method described by Buckner et al was used as a covariate(166).

2.8 Hyperinsulinemic euglycemic glucose clamp

Insulin sensitivity was assessed after a 10hr overnight fast using the hyperinsulinemic euglycaemic clamp technique. Insulin was infused at a fixed-rate of 40 mU/m^2 body surface area/minute for the duration of the clamp (90min) in order to achieve a plateau (steady-state) insulin level about 100 µU/mL above basal concentration. Blood glucose level was measured every 5min using the glucose oxidase method (Yellow Spring glucose analyzer, Ohio, USA). The infusion rate of the 20% dextrose solution was adjusted to maintain a constant blood glucose level at approximately 90mg/dL (5mmol/L) throughout the clamp. Under these conditions, hepatic glucose production will be totally suppressed. Insulin sensitivity index (ISI) (M/I) was calculated using the mean glucose infusion rate (M) and steady state insulin concentration (I) during the final 30min of the clamp(167).

2.9 Biochemical analyses

Venous blood samples were drawn from all subjects in the morning after a 10-hour overnight fast. Blood samples were collected according to recommendations of the individual assay kits' manufacturers. Serum and plasma samples were frozen and stored at -80°C until the tests were performed. Serum insulin was measured using a chemiluminescence assay (ADVIA Centaur analyzer, Siemens Healthcare Diagnostics). Plasma glucose was measured using an automated analyzer (ADVIA 2400; Bayer Diagnostics, New York, USA).

2.10 Enzyme-linked immunosorbent assay (ELISA)

Plasma and serum levels of adipokines were determined by enzyme-linked immunosorbent assay (ELISA). Serum leptin, fibroblast growth factor 21 (FGF21), resistin, monocyte chemoattractant protein 1 (MCP1) and rentinol binding protein 4 (RBP4) levels were measured using ELISA kits(Millipore, Billerica, MA). Plasma total adiponectin was measured by adiponectin (multimeric) ELISA kit (Alpco Diagnostics, Salem). Serum apelin levels were determined by ELISA kit(Human Apelin ELISA kit Phoenix Pharmaceuticals, Belmont, CA). Protocol was as follows: Add 50 ml/well of standard, sample, or positive control, 25 ml primary antibody and 25 ml biotinylated peptide. Incubate at room temperature for 2 hours. Wash immunoplate 4 times with 350 ml/well of 16assay buffer. Add 100 ml/well of streptavidin-horseradish peroxidase (SA-HRP) solution and incubate at room temperature for 1 hour. Wash immunoplate 4 times with 350 ml/well of 16assay buffer. Add 100 ml/well of Tetramethylbenzidine (TMB) substrate solution and incubate at room temperature for 1 hour. Terminate reaction with 100 ml/well of HCI.

Read absorbance OD at 450 nm and calculate results.

2.11 Muscle Biopsies

Percutaneous muscle biopsy was obtained from the vastus lateralis under local anaesthesia in individuals who provide informed consent for this procedure. One specimen was aliquoted immediately into phosphate buffered saline (PBS) for myoblast cultures. The other samples will be frozen in liquid nitrogen and stored at -80 °C. Muscle tissue was minced and digested with 0.2% collagenase type 1A (C5894; Sigma-Aldrich, St. Louis, USA) for 90 minutes at room temperature with shaking at 750 rpm, after which the cultures were pelleted down and passed through the 100 μm filter to remove undigested tissue. The cells were then re-suspended in Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% fetal bovine serum (FBS, Invitrogen, Carlsbad, USA), 10% horse serum (HS) (Invitrogen), 1% penicillin-streptomycin (PS) (Invitrogen) and 1% chick embryo extract (C3999; US Biologicals, USA) and enriched for myoblasts by pre-plating on uncoated plates for 3hrs. After 3 hours of attachment, the supernatant having cells were preplated on to matrigel (Becton Dickinson)-coated plates and myoblast cultures were maintained at 37°C and 5% CO_2. Upon reaching 90% confluency, primary myoblasts will be induced to differentiate in DMEM supplemented with 2% HS and 1% PS for 7 days prior to experimentation. Myotubes will be fully formed after 7 days of differentiation and were harvested.

2.12 Mitochondrial respiration

Mitochondrial bioenergetics was measured in primary human myotubes ex vivo using a Seahorse XF24 Analyzer (Seahorse Biosciences, North Billerica, MA). Myoblasts were seeded on XF24 tissue culture plates at 20 000 cells/well in growth medium. Differentiation of myoblasts into myotubes was initiated at approximately 90% confluence, by switching to low-serum differentiation media containing DMEM, 2% HS and 1% PS. Myotubes were studied after 7 days of differentiation when myoblasts had fused to form multinuclear elongated myotubes. On the day of the assay, myotubes were then incubated for 45 min at 37°C at ambient CO_2 in XF Assay Medium (Seahorse Biosciences) (pH 7.4) containing 4 mM glutamine, 1 mM pyruvate and 25 mM glucose. Oxygen consumption rate (OCR) was measured by monitoring the concentrations of dissolved oxygen by the XF24 Analyzer solid-state sensor probes above the cell monolayer. Baseline OCR ($OCR_{Baseline}$) was measured 3 times for 4 min each separated by a 2 min wait and a 2 min mix. Following the measurement of basal respiration, oligomycin (1uM) (Sigma–Aldrich, Oakville, ON, Canada) was injected into each well, followed by 3 cycles of: 2 min mix, 2 min wait and 4 min measurement to measure proton leak dependent respiration ($OCR_{oligomycin}$). Oligomycin inhibits ATP synthesis by blocking the proton channel of the F_o portion ATP synthase, resulting in protons returning to the matrix through leak pathways, therefore measuring the proportion of oxygen used for ATP turnover and proton leak. Next, a mitochondrial inner membrane uncoupler, carbonyl cyanide p-trifluoro methoxyphenylhydrazone (FCCP) (0.8 mM) (Sigma–Aldrich, Oakville, ON, Canada) was injected into each well, followed by 3 cycles of: 2 min mix, 2

min wait and 2.5 min measurement to measure maximal respiration (OCR_{FCCP}). FCCP produces a collapse in the proton gradient across the mitochondrial inner membrane, hence measuring the capacity of the electron transport chain (ETC). Finally, a mixture of rotenone (1 μM) and antimycin A (1μM) that blocks respiratory chain complexes I and III, was injected into each well, followed by 3 cycles of: 2 min mix, 2 min wait and 3 min measurement to measure non-mitochondrial respiration ($OCR_{R/A}$). Parameters of mitochondrial bioenergetics were calculated as follows: 1) Basal OCR = $OCR_{Baseline} - OCR_{R/A}$, 2) ATP turnover = $OCR_{Baseline}$ - $OCR_{Oligomycin}$, 3) Proton leak = $OCR_{Oligomycin} - OCR_{R/A,}$ 4) Maximal respiration = $OCR_{FCCP} - OCR_{R/A}$, 5) spare respiratory capacity = $OCR_{FCCP} - OCR_{Baseline}$ (Figure 2.1) and 6) Non-mitochondrial respiration ($OCR_{R/A}$). A total of 6 technical replicates were performed for each subject.

The strength of measuring mitochondrial function in intact muscle fibers as opposed to isolated mitochondria is the greater physiological relevance. In intact cells, mitochondrial structure and membrane are intact, interactions between mitochondria and rest of the cell are preserved and mitochondria are exposed to a relevant mix of substrates and ions(168). In contrast, isolated mitochondria in vitro are typically exposed to non-physiological conditions. First, isolated mitochondria in vitro are exposed to oxygen concentrations that are much higher than what is present in cellular microenvironment in vivo. Furthermore, respiration and ATP synthesis rates are measured in response to substrate and ADP concentrations that are saturating and dramatically exceed

levels that are endogenous to the cell(169). The disadvantages however are that the mitochondria are not directly accessible to the full range of substrates and inhibitors, and the complexity of cytoplasmic metabolism must be considered together with the presence of separate pools of adenine nucleotides, nicotinamide nucleotides and calcium in the cytoplasm and mitochondrial matrix(168).

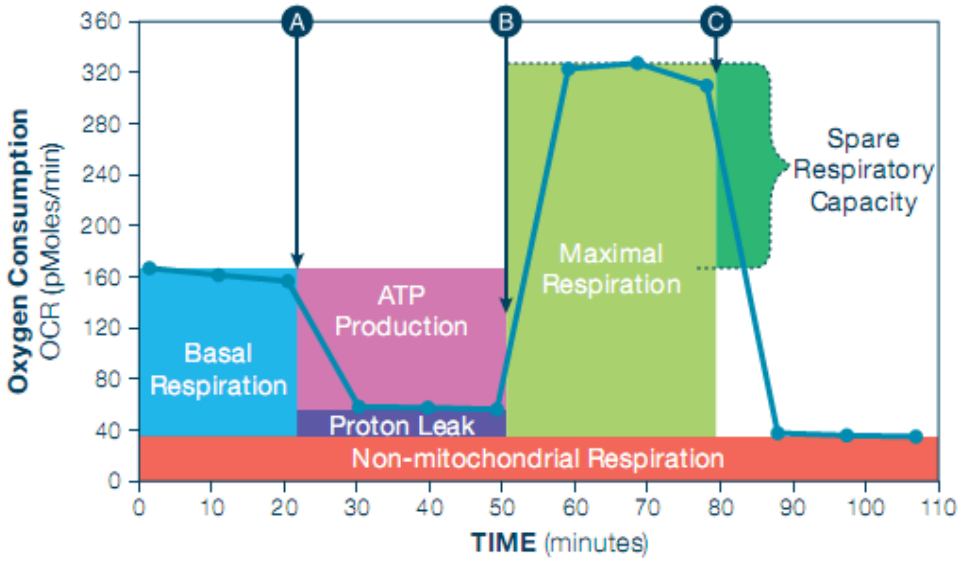


Figure 2.1 Mitochondrial bioenergetics profile

2.13 Quantification of mRNA

2.13.1 Total RNA isolation

Total RNA was extracted from myotube cultures with RNeasy plus mini kit (Qiagen). Briefly, 600 µl of Buffer RLT Plus and 6 µl of β-mercaptoethanol (β-ME) was added to the 10 cm cell-culture dish. Lysate was homogenized by vortexing for 1 min. Lysate was pipetted directly into a QIAshredder spin

column and centrifuged. The homogenized lysate was transferred to a gDNA Eliminator spin column and centrifuged. 600 µl of 70% ethanol was added to the flow-through which was transferred to an RNeasy spin column. The column was washed with buffers and RNA was eluted out in 30 µl of RNase-free water.

2.13.2 Reverse transcription (RT)

cDNAs were synthesized from 1 µg of total RNA in a 20 µl volume using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 2 X RT master mix reaction was prepared with the following components and mixed with 10 µl of RNA sample.

10 X RT Buffer	2.0 µl
25 X dNTP Mix (100 mM)	0.8 µl
10 X RT Random Primers	2.0 µl
MultiScribe Reverse Transcriptase	1.0 µl
RNase free water	4.2 µl

RT was then performed with the following conditions: 10 min at 25 °C, 120 min at 37°C and 5 min at 85 °C. cDNA was diluted to 100 µl final volume and used immediately or stored at -20 °C.

2.13.3 Quantitative real-time PCR (RT-PCR)

RT-PCR reactions were performed in duplicates for each sample in a total reaction volume of 10 ul with the following components.

2 X Quantifast SYBR Green PCR Master Mix (Qiagen)	5 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
RNase-free water	1 µl
cDNA (10 ng/µl)	2 µl

The reaction conditions were 5 min at 95°C for the initial heat activation step, followed by 40 cycles of 10 s denaturation at 95°C and 30 s annealing and extension at 60 °C. Dissociation curves were performed at the end of all runs to ensure specificity of primers. The relative expressions of respective genes were normalized to GAPDH and calculated using the comparative Ct quantitation method (2^ -ddCT). All primers were designed to span two consecutive exons to avoid co-amplification of genomic DNA, which may contaminate the RNA preparation. The complete list of primers is listed in Table 2.1. Reactions were performed using a Prism 7000 Sequence.

Table 2.1 List of primers used for real time PCR detection of gene expression in primary human myotubes.

Gene	Forward primer/Reverse primer
Myo	CGCCAGGATATGGAGCTACT GAGTGCTCTTCGGGTTTCAG
Myosin heavy chain	GTATAAGCCCGAGGTGGTGA GTCCCCTGTATTTTGCCAGA
UCP3	AGCCTCACTACCCGGATTTT CGTCCATAGTCCCGCTGTAT
UCP2	TGCTGAGCTGGTGACCTATG GGGCATGAACCCTTTGTAGA
ANT1	GCCAACGTGATCCGTTACTT ATGATACAGTCGCCCAGACC
CPT1B	CATCAAGAATGGCATCCTCA ATCCACCCATGGTAGCAGAG
HADHA	CAAATCAGAAGTGCCGTCCT CCAGCAAAACTTCAGGGGTA
COX15	CCTGCTGCCTACTTTTGGAG GTGGGTTTCAGGCAACTTGT
NDUFB4	GACCCAGCCGAATACAACAT CACAGAGCTCCCATGAGTGA
ATP5J2	ATCTTGATGCGGGACTTCAG AGATGCTCCCCTTCTTCACA
CS	CCATCCACAGTGACCATGAG CTTTGCCAACTTCCTTCTGC
IDH3G	TTTGAAGAGGTGCACGTGAG ATGCTCCAGGCTGCTGTACT
PGC1A	GCAGTCCTCACAGAGACACT AGCCTCATTGTCAGTGGTCA
ACC1	TCCTTGTCACCTGCTTCTGT TTTCTTTCTGTCTCCGCCCT
FASN	TCAAAGGACCAAGCATTGCC GGCATTCAGAATCGTGGCAT
GAPDH	CATGTTCGTCATGGGTGTGA TGGACGTGGTCATG AGCCT

2.14 Quantification of protein

2.14.1 Protein lysate preparation

Cell cultures were lysed with RIPA buffer with cocktail of protease inhibitor, phosphatase inhibitor and EDTA (Pierce).

2.14.2 Protein concentration determination

Protein concentrations were determined using the Bio-rad Bradford protein assay. Briefly, eight serial dilutions of a standard BSA solution were prepared, starting with 1.0 mg/ml. 10 µl of the BSA standards and protein samples were added into the wells of a 96-well microtiter plate. The dye reagent was diluted (1 part Dye reagent concentrate with 4 parts distilled water) and filtered through 0.45 µM filter. 200 µl of the dye was then added to each well and incubated at room temperature for 5 min. Absorbance was measured at 595 nm. The standard curve was constructed by plotting the known BSA concentration. Protein concentrations were determined by comparison with the BSA standard curve.

2.14.3 SDS-PAGE separation of protein

Typically, 10-25 ug of protein samples were run per well and were mixed with 5X Laemmli buffer and β-mercaptoethanol. The samples were denatured by boiling for 10 min at 95°C before loading into the wells of 10% SDS-PAGE gel. The gel was run in 1 X SDS/Glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH 3) at 100 V until the dye front is approximately at the bottom of the gel.

2.13.4 Western Blot

Proteins resolved by SDS-PAGE were transferred to PVDF membranes (Bio-Rad). The membranes were then blocked in 5.0 % milk in PBST (10 mM Tris HCl, 100 mM NaCl, 0.02% Tween 20) and incubated with appropriate primary antibodies overnight at 4ºC with shaking. Primary antibodies used were FTO (Abcam, ab92821), phospho-Akt (Ser473) (Santa Cruz), UCP3 (Abcam, ab3477), ANT1 (Santz Cruz, sc-11433), COX IV (Cell Signalling, 4844), citrate synthase (Santa Cruz, sc-390693) with GAPDH (Cell Signalling) as a loading control. Membranes were washed with PBST and incubated with either anti-rabbit IgG Horseradish Peroxidase (HRP) conjugate (Bio-Rad) or anti-mouse IgG HRP conjugate (Bio-Rad) for 1 h at room temperature. Primary and secondary antibodies incubations were done in blocking buffer. The HRP activity was detected using Western Lightning Chemiluminescence Reagent Plus (NEL104; PerkinElmer Life Sciences, Wellesley, MA) and exposure to autoradiography film. Blots were quantified by densitometric analyses using Image J.

2.13.5 Medias and Buffers

1 X PBS, pH 7.4 (Invitrogen, Singapore)

- KH_2PO_4 (1.76 mM)
- Na_2HPO_4 (10.4 mM)
- NaCl (137 mM)
- KCl (2.7 mM)

Tris-buffered saline

- Tris-HCL, pH 7.5 (50 mM)
- NaCl (150 mM)

5 X Reducing Laemmli buffer (Biorad, Singapore)

- Tris-HCL, pH 6.8 250 mM
- Glycerol 50% (v/v)
- SDS 10% (w/v)
- Bromphenol blue 0.05% (w/v)
- B-mercaptoethanol (0.5 ml in 10 ml of sample loading buffer)

10X SDS-PAGE electrophoresis buffer

- Tris base 250 mM
- Glycine 2.5 M
- SDS 1% (w/v)

SDS-PAGE stacking gel

- dH20 (3.05 ml)
- 0.5 M Tris-HCL, pH 6.8 (1.25 mL)
- 10% (w/v) SDS (50 µl)
- 30% Acrylamide/Bis solution 29:1 (3.3%) (0.65 ml)

- 10% APS (25 µl)
- TEMED (5 µl)

SDS-PAGE separating gel (10%)

- dH20 (3.17 ml)
- 1.5 M Tris-HCL, pH 8.8 (2.5 mL)
- 10% (w/v) SDS (100 µl)
- 30% Acrylamide/Bis solution 29:1 (3.3%) (4.16 ml)
- 10% APS (50 µl)
- TEMED (5 µl)

10X Western Blot transfer buffer

- Tris base 25 mM
- Glycine 192 mM
- Methanol 15% (w/v)

PBST buffer

- 1 X PBS buffer with 0.05 % Tween 20

Blocking buffer

- PBST with 0.05% non-fat milk

Chapter 3 : Validation of prediction equations for resting energy expenditure in Singaporean Chinese men

3.1 Introduction

The accurate determination of total energy expenditure (TEE) is important for establishing dietary intake goals in weight management of both normal weight and obese individuals and nutritional management of hospitalized patients to avoid the adverse effects of overfeeding and underfeeding. It is also used in estimating the energy requirements of a population. Since the routine use of indirect calorimetry (the criterion standard for measuring REE) is not feasible in daily clinical practice, predictive equations are often used to estimate REE(6). The validity of any predictive equation is crucial as REE is often used as the predictor of TEE as it accounts for 60-70% of TEE for people leading a sedentary lifestyle(8). Any bias in REE assessment would be amplified when it is multiplied by the factor that is used to estimate the TEE of a population or individuals. The Food and Agriculture Organization/World Health Organization/United Nations University (FAO/WHO/UNU) 1985 predictive equation developed using data from Schofield and colleagues have been adopted as a basic reference for the development of energy requirements in Singapore(77). However, it is unclear whether this equation can be applied successfully to the Singaporean population. The FAO/WHO/UNU equations, developed using predominantly European subjects, seemed to be less accurate in predicting REE in Asian populations(131, 170). In a study by Ismail et al., FAO/WHO/UNU equations overestimated the REE of adult Malays, Chinese, Indians and Dayaks living in Malaysia by 13% in male and 9% in female subjects(171) Recently, Tseng et al. reported that the measured REE was

significantly lower than REE calculated by the equation by 271 ± 311 kcal/day in Chinese subjects living in Taiwan(172). Researchers have also queried the continued applicability of the FAO/WHO/UNU equations in our modern population, with changes in body size and composition, physical activity level and diet (2). Several studies have shown that these predictive equations overestimated REE in Caucasian as well as in Asian populations(18, 173). Muller et al. reported that the WHO equations overestimated REE from the measured values by up to 14.3% in males and 7.5% in females in Germany(18). According to Piers et al., measured REE of Australian men and women were significantly lower than the predicted REE using FAO/WHO/UNU equations by 406 ± 513 kJ/day and 124 ± 348 kJ/day respectively(174). No validation studies on prediction equations for REE have been conducted in Singapore. Therefore, it is crucial to identify the most accurate equation appropriate for predicting energy needs in this population. The objective of this study was to evaluate the validity of FAO/WHO/UNU equation and other commonly used prediction equations, Harris Benedict, Mifflin et al and Owen et al from the literature in a sample of healthy Singaporean Chinese men.

3.2. Materials and Methods

3.2.1 Subjects

A total of 96 healthy Singaporean Chinese men participated in this cross-sectional study. Inclusion and exclusion criteria of subjects were described in Section 2.2.

3.2.2 Anthropometry

Body weight and height was measured as described in section 2.3 BMI was calculated as weight divided by height squared (kg/m^2).

3.2.3 Measurement of resting energy expenditure

REE was measured continuously by open-circuit indirect calorimetry using a ventilated hood system (Quark CPET, COSMED, Italy) for 60 min as described in Section 2.4. REE was calculated from VO_2 and VCO_2 by the Weir formula(163). In addition to measured values, REE was predicted by the FAO/WHO/UNU, Harris-Benedict, Owen et al and Mifflin et al equations (Table 3.1).

Table 3.1 Prediction equations for resting energy expenditure in males.

Prediction Equation	Formula
Harris Benedict(1919)	REE= 66.473+ 13.7516*Weight +5.0033*Height -6.755*Age
Owen(1986)	REE=879 +10.2*Weight
Mifflin St Jeor (1990)	REE=10*Weight +6.25*Height -5*Age +5
FAO/WHO/UNU (1985)	18-29 yr: REE= 15.3*W + 679 30-59 yr: REE= 11.6*W + 879

REE= resting energy expenditure in kcal/day. All equations use weight in kg,height in centimeters and age in years

3.2.4 Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 20(175). Pearson's correlation coefficients were used to examine the association between measured and predicted REE for each of the predictive equations. Measured and predicted REE were compared at a group level using student's paired t test. Individual prediction accuracy was defined as the percentage of subjects that had an REE predicted within ±10% of REE measured(176). A prediction below 90% of REE measured was classified as under-prediction and a prediction above 110% of REE measured was classified as over-prediction(177). Bland and Altman plots (178) were used to determine agreement both graphically and numerically between measured REE and predicted REE by calculating mean bias (difference between measured and predicted REE) and limits of agreement (mean bias ± 2 SD). For good agreement, it is expected that bias will be close to zero, clinically acceptable limits of agreement that are within ±10% of measured REE and that there is no clear evidence of a relationship between difference and mean of measured and predicted REE Values are given as Mean ± SD The threshold for significance in all tests was set at $P < 0.05$ (two sided).

3.3 Results

3.3.1 Subject characteristics

We studied 96 Chinese men with mean age of 28.4±6.0 years, weight of 69.4±10.3 kg, height of 172±5.8 cm and BMI of 23.4±2.9 kg/m2.

3.3.2 Mean differences and correlations between measured and predicted resting energy expenditure

Significant correlations were found between measured REE and REE derived from the FAO/WHO/UNU equation(R = 0.68), Harris-Benedict equation (R = 0.71), Mifflin equation (R = 0.70) and Owen equation(R = 0.67) (Table 3.2). However, the FAO/WHO/UNU, Harris-Benedict and Mifflin equations significantly overestimated mean measured REE by 120 ± 140 kcal/day, 96 ± 137 kcal/day and 38 ± 136 kcal/day respectively. The Owen equation did not show a significant mean bias in the prediction of REE.

Table 3.2 Differences and Pearson correlation coefficients between measured and predicted resting energy expenditure

	REE		Difference		95% CI		R	Limits of agreement
	Mean	S.D	Mean	S.D	Lower	Upper		
Measured	1594	191						
FAO/WHO/UNU	1714	137	120*	140	91	148	0.679*	-161 to 400
Harris Benedict	1689	158	96*	137	68	123	0.705*	-179 to 371
Mifflin	1632	126	38*	136	11	66	0.700*	-235 to 311
Owen	1587	105	-7 NS	144	-36	22	0.666*	-295 to 281

NS, not significant

*P<0.05

3.3.3 Bland Altman analyses

The individual differences between measured and predicted values of REE plotted against the average of measured and predicted values are shown in Figure 3.1. The limits of agreement were wide for all equations. The individual differences between measured and predicted REE ranged from −161 kcal/day to 400 kcal/day.

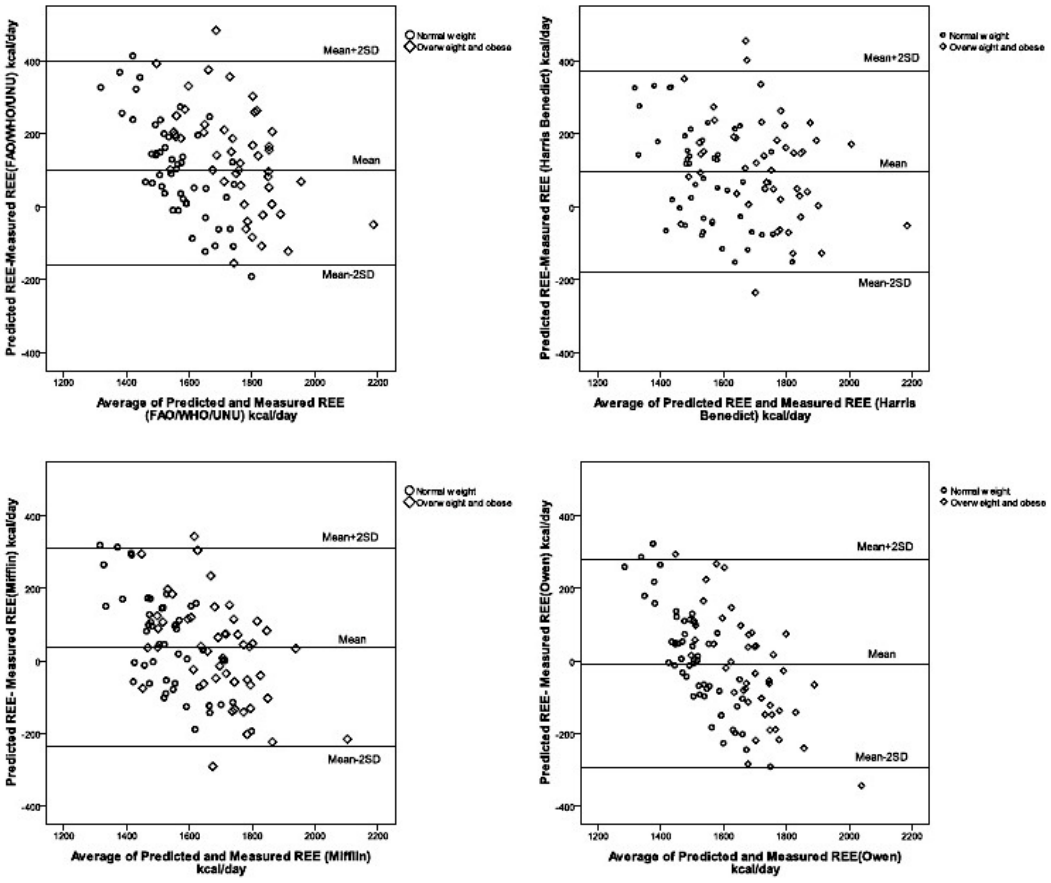


Figure 3.1 Bland Altman plots for comparison of agreement between resting energy expenditure measured by indirect calorimetry and predicted resting energy expenditure derived from prediction equations.

3.3.4 Percentage of accurate predictions, under-predictions and over-predictions

Focusing on the distribution of individual measured REE values revealed that Mifflin equation had the highest percentage REE predictive accuracy, with 75% of the subjects falling within ±10% of measured REE followed by the Owen equation with an individual prediction accuracy of 73% (Table 3.3). Compared to the Mifflin equation that more commonly overestimated REE than underestimated it, the Owen equation had similar numbers in which REE was over- or underestimated. REE could not be accurately predicted by the FAO/WHO/UNU and Harris-Benedict equations in more than 30% of the subjects.

Table 3.3 Percentage of subjects with accurate predictions, under-predictions and over-predictions.

Equation	Accurate prediction (%)	Under-prediction (%)	Over-prediction (%)
FAO/WHO/UNU	60.4	1	38.5
Harris Benedict	66.7	1	32.3
Mifflin	75	5.2	19.8
Owen	72.9	14.6	12.5

3.4 Discussion

In our study, the measured REE was significantly lower than that predicted by the FAO/WHO/UNU, Harris-Benedict and Mifflin equations by 7.5%, 6.0% and 2.4% respectively, in a group of adult Singaporean Chinese men. However, the Owen equation provided valid estimates of REE with only slight underestimation (0.4%). Several researchers have also reported overestimation of REE in Asians using FAO/WHO/UNU and Harris-Benedict equations, and found percentage differences comparable to those of our study(8). According to Nhung et al, predicted values using the FAO/WHO/UNU equations were significantly higher than measured REE by 8.5% and 5.4% respectively in Asian subjects(6). In a study by Liu et al, the Harris Benedict equation over-predicted measured REE by 8% in Chinese. On the other hand, the Liu equation that was derived from Chinese adults, predicted the REE of Asians more accurately(77). These are similar to the 7.5% (FAO/WHO/UNU equation) and 6.0% (Harris Benedict equation) observed in our study. It has been suggested that such overestimation of REE by these prediction equations in Singaporean Chinese may be largely explained by ethnic differences in REE related to body composition(86, 131, 179). The Schofield database was dominated by Italian data that constituted 3388 out of a total of 7173 REE data points(164, 170). Hayter and Henry further reported that Italian subjects had a higher REE per kilogram of body weight compared to other population groups (North Europeans, Americans, Indians and Asians) in the Schofield database(180). The Harris-Benedict equation was also developed using predominantly white subjects(172). On the other hand, the Owen equation was

developed from a group of Caucasian, African-Americans and Asian male subjects, and hence, may be more accurate in predicting energy needs in Singaporean Chinese men(2). In addition, the subjects used in the Schofield study were primarily young people with relatively high activity levels. Labourers and miners, Italian military, Neapolitan police, Italian navy and Burmese hill dwellers made up the largest groups of men in the Schofield database(18) . In contrast, the subjects used in our study were sedentary. Poehlman et al. reported a higher REE in physically active individuals compared to those with sedentary lifestyles, even after adjustment for body composition(181). At the individual level, Bland Altman plots of all equations showed lack of agreement with measured REE with wide limits of agreement. Errors of REE measurement may be introduced by air leaks, incorrect calibration of the calorimeter, involuntary alternating periods of hyper and hypoventilation, fluctuating levels of fractional inspired O_2 concentration, or acid-base disturbances(182). Although REE measurements were performed under strictly standardized conditions, biological intra-individual variation in REE might also be due to variations in eating and activity pattern in the days before the REE measurements. Limits of agreement within ±10% of measured REE are considered clinically acceptable as intra-individual variation in REE was reported to be 2-10% explained by methodological and biological variability in REE(183). However, in our study, only 70% of subjects had limits of agreement < 10%. Furthermore, for all equations, Bland Altman plot showed that bias was not consistent across the range of REE values because bias correlated significantly with the mean of measured and predicted REE (data not shown). Predicted REE typically deviated more profoundly in the

lower and upper REE ranges, with the equations systematically over-estimating at lower REE and underestimating at high REE. This was also observed in other studies(184, 185). Considering that the average REE was about1600 kcal/day, an error of 10% would translate into a 160 kcal/day deviation from true REE. However, the Owen equation demonstrated individual differences between measured and predicted energy values that ranged from −295 to 281 kcal/day, which is about −19 to 18% of REE. If these equations were closely followed to establish energy intake in a controlled setting over the long term, energy needs could be over-predicted or under-predicted by the physiologic weight change equivalent of 0.3 kg/week or 14.4 kg/year. This is especially detrimental in the clinical setting where over-feeding or underfeeding may have adverse effects such as electrolyte imbalance and gastrointestinal problems (186). And in weight management for overweight and obese people, prescribed dietary plans might be ineffective in producing weight loss. A limitation of our study is that the REE measurements were made on an outpatient basis and could not be performed earlier in the morning. However, subjects were allowed to rest comfortably for at least 15 min before measurement, during which they became acclimated to the environment, therefore ensuring resting conditions. Also, the standard deviation found in our study is comparable to other studies that had REE measurements conducted at 0700 h with the subjects staying overnight in the facility(36). Therefore this limitation did not add to the variability of the REE measured and is unlikely to affect the results reported in this paper

These findings show that the FAO/WHO/UNU, Harris-Benedict and Mifflin predictive equations significantly overestimated energy needs for Singaporean

Chinese men, therefore usage of these equations may put them at a greater risk for developing obesity, especially in overweight people. We recommend the Owen equation for estimation of energy needs in Singaporean Chinese adult males at a group level due to small mean prediction error. However, the individual errors of the equation are too high to be of practical use in individuals, in situations where accurate estimation of REE is clinically relevant (such as when prescribing nutrition in the setting of treatment for the critically ill or during the treatment of obesity). Although the Owen equation predicted REE accurately in 73% of the subjects, it displayed wide limits of agreement and directional bias was observed across the range of REE. These wide limits of agreement were especially prominent in those with more extreme levels of REE. Therefore, in individuals where a precise determination of REE is indicated, measurement by indirect calorimetry instead of the prediction of REE using equations is highly recommended.

Chapter 4 . Smaller size of high metabolic rate organs explains lower resting energy expenditure in Asian-Indian than Chinese men.

4.1 Introduction

Obesity is a global epidemic, as the prevalence of overweight and obesity is increasing not only in developed but also in developing countries (3). It is estimated that, by 2030, about 58% of the world population will be obese (Body mass index, BMI $\geq$ 30 kg/m^2)(187). The population in Singapore comprises three major ethnic groups: Chinese, Asian Indians and Malays. They live in a small geographic area that is highly urbanized. The prevalence of obesity (defined as a BMI>=30 kg/m2) has doubled in the last decade from 6.9% in 2004 to 10.8% in 2010. This increase has occurred primarily 30-39 years age group(188). Among the ethnic groups, obesity prevalence is disproportionately higher in Malays (24.0%) and Asian-Indians (16.9%) than Chinese (7.9%). We have previously shown that over an 8-year period, Asian Indians and Malays gained the most weight compared to Chinese(189)

Obesity is the result of an energy imbalance where chronic energy intake exceeds energy expenditure, resulting in adipose tissue accumulation(190). In Singapore, caloric intake has been estimated to be lower in the Chinese (2367 kcal) compared to Asian-Indians (2499 kcal) and Malays (2522 kcal) and. However, ethnic differences in resting energy expenditure (REE) may also be a contributory factor. REE is a major component of total energy expenditure (TEE) and accounts for 60-70% of TEE for people leading a sedentary lifestyle(191). In several adult populations, reduced REE was reported to be a

predisposing risk factor for long-term weight gain & obesity(14, 15). REE has been shown to differ between ethnic groups in studies performed in other populations. Many studies showed lower REE in African-American women(23, 192), men(21) and children(193, 194) than their White counterparts, independent of body weight or body composition. Soares and colleagues reported lower REE in Indians when compared with Westerners matched for body weight, but difference was no longer significant after adjusting for body composition(195).

The primary aim of this study was to explore the association between ethnicity and REE in Chinese, Asian Indian and Malay men living in Singapore and to determine the role of potential contributors to these ethnic differences including fat free mass (FFM), fat mass, mass/volume of high metabolic rate organs (HMRO) (represented by brain volume and trunk FFM). The secondary aim is to examine whether inter-individual variation in circulating concentrations of adipokines such as leptin, adiponectin, fibroblast growth factor 21 (FGF21), apelin, resistin and monocyte chemoattractant protein 1 (MCP1), help to account for inter-individual variation in REE once the effects of FFM and fat mass had been accounted for.

4.2 Materials and methods

4.2.1 Subjects

244 healthy men of different ethnicities (100 Chinese, 70 Indian and 74 Malay) were involved in this cross-sectional study. Details are described in Section 2.2

4.2.2 Anthropometry & body composition

Body weight and height were measured as previously described in Section 2.3 Whole body and regional (trunk, arms and legs) lean body mass (LBM), fat mass and bone mineral content (BMC) were measured by dual-energy x-ray absorptiometry (DEXA; Hologic Discovery Wi) as previously described in Section 2.3.

4.2.3 Measurements of resting energy expenditure

REE was measured in the early morning after the subjects had fasted for at least 10 hours, by open-circuit indirect calorimetry using a ventilated hood system (Quark CPET, COSMED, Italy) for 60 minutes as previously described in Section 2.4. They were instructed to transport themselves to the hospital in a vehicle to avoid undue exertion. They were required to undergo a 12 h overnight fast and refrain from intensive physical activity for 24 h prior to measurement. REE was calculated from VO_2 and VCO_2 by the Weir formula(163)

4.2.4 Brain volume

High-resolution images of the brain were acquired by magnetic resonance imaging using a T1- weighted MP–RAGE sequence as described in Section 2.7.

4.2.5 Enzyme-linked immunosorbent assay (ELISA)

Venous blood samples were drawn from all subjects in the morning after a 10-hour overnight fast. Blood samples were collected according to recommendations of the individual assay kits' manufacturers. Adipokines were measured by enzyme-linked immunosorbent assay (ELISA) as previously described in Section 2.10. Serum leptin was measured by ELISA kit (Millipore, Billerica, MA) with intra-and inter-assay coefficients of variation (CVs) of 3.3% and 9.4% respectively**.** Serum total adiponectin was measured by ELISA kit (Alpco Diagnostics, Salem). Intra-and inter-assay CVs for total adiponectin were 3.3% and 6.5% respectively. Serum apelin levels were determined by ELISA kit (Human Apelin ELISA kit Phoenix Pharmaceuticals, Belmont, CA). Intra-and inter-assay CVs were 14.2% and 56.5% respectively. Serum FGF21, resistin and MCP1 levels were measured by ELISA kit (Millipore. Billerica, MA). Intra-and inter-assay CVs for FGF21 were 3.8% and 7.4% respectively. Intra-and inter-assay CVs for resistin were 4.5% and 14.1% respectively. Intra-and inter-assay CVs for MCP1 were 4.5% and 11.7% respectively.

4.2.6 Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics for Mac Version 20.0; Armonk, NY: IBM Corp(196). All variables were checked for normal distribution, and variables with skewed distributions were log transformed to satisfy conditions of normality All statistical tests were two-sided, and a P value<0.05 was considered statistically significant. All values were given as mean± standard error. A single regression model in which REE was regressed on total LBM was used to identify outliers within each ethnic

group. There were 9 participants with standardized residual values above 2.0 or less than - 2.0 and these were excluded from subsequent analyses. Analysis of variance (ANOVA) and analysis of covariance (ANCOVA) were used for comparisons of continuous variables between ethnic groups with Tukey corrections applied for group comparisons. Multiple regression analyses were used to assess the contribution of independent variables (total FFM, fat mass, brain volume, trunk FFM and limb FFM) to the association between REE and ethnicity. We removed the effects of FFM and fat mass on REE to derive a residual variance using ANCOVA. We sought to explore how much of this residual variation could be explained by individual differences in circulating adipokines by using Pearson correlation analysis. ANCOVA models were constructed to test for ethnic differences in the relationship between residual REE and each adipokine with residual REE as the dependent variable, ethnicity as a factor and adipokine as a covariate. The cross-product interaction term “ethnicity X adipokine” was included as an independent variable

4.3 Results

4.3.1 Subject characteristics

Ethnic differences were observed in relation to anthropometry, body composition and brain volume (Table 4.1). Chinese were older than Asian-Indians. Body weight and BMI were significantly higher in Asian-Indians and Malays and than Chinese. After adjustment for age and body weight, trunk FFM was significantly lower and limb FFM was significantly higher in Asian-Indians than Chinese even though total FFM was similar. Asian Indians had greater fat mass and lower brain volume than Chinese before and after adjustment for age and body weight. Ethnic difference in brain volume persists even after adjustment for differences in eTIV between Chinese and Asian-Indians. Unadjusted REE was not significantly associated with ethnicity. After adjusting for age and body weight, REE was significantly lower in Asian-Indians (1551± 16 kcal/day) than in Chinese (1624± 14 kcal/day) but the difference in REE between Malays (1589± 16 kcal/day) and Chinese was not significant.

Table 4.1 Anthropometric characteristics, body composition and resting energy expenditure of study participants by ethnicity

	Chinese (N=96)		Asian-Indian (N=68)		Malay (N=71)			
	Mean	SD	Mean	SD	Mean	SD	P value [a]	P value [b]
Age (y)	28.39	5.99	25.96	4.90	27.38	4.88	**0.018**	
Weight (kg)	69.37	10.26	74.60	10.90	73.41	11.77	**0.006**	**<0.001***
Height (cm)	172.03	5.83	173.44	5.77	171.35	6.70	0.120	0.138*
BMI (kg/m^2)	23.39	2.88	24.78	3.20	24.98	3.56	**0.002**	**<0.001***
Fat Mass (kg)	14.63	5.27	17.93	6.52	17.34	7.41	**0.002**	0.081
Total FFM (kg)	52.94	6.52	55.02	5.93	54.15	5.92	0.10	0.145
Trunk FFM (kg)	24.80	3.09	25.08	2.96	24.86	2.78	0.83	**<0.001**
Limb FFM (kg)	24.10	3.44	26.21	3.01	25.31	3.06	**<0.001**	0.117
Brain volume (cm^3)	1.20	0.08	1.14	0.10	1.18	0.10	**0.001**	**<0.001**
eTIV	1.42	0.16	1.36	0.13	1.40	0.16	**0.051**	0.134
REE (kcal/day)	1594	191	1579	164	1604	181	0.71	**0.004**

[a] P_{ANOVA} value for comparison between ethnic groups

[b] P_{ANOVA} value for comparison between the ethnic groups adjusted for age and body weight

* adjusted for age only and not body weight

4.3.2 Correlations between resting energy expenditure with anthropometry, body composition and brain volume

Univariate correlation analyses showed that REE was positively associated with weight, BMI, fat mass, total and regional FFM across all ethnic groups (Table 4. 2). Compared with fat mass, REE correlated more strongly with total FFM and regional FFM distribution. The positive association between REE and brain volume was evident in Malays but not in Chinese and Asian-Indians. The correlation with age was not significant.

Table 4.2 Pearson correlation coefficients between resting energy expenditure with age, anthropometry and body composition by ethnic group.

	Chinese (N=96)		Asian-Indian (N=68)		Malay (N=71)	
	R	P Value	R	P Value	R	P Value
Age (y)	-0.105	0.31	0.202	0.099	0.14	0.244
Weight (kg)	0.666	**<0.001**	0.688	**<0.001**	0.666	**<0.001**
Height (cm)	0.463	**<0.001**	0.325	**0.007**	0.412	**<0.001**
BMI (kg/m2)	0.536	**<0.001**	0.592	**<0.001**	0.523	**<0.001**
Fat Mass	0.37	**<0.001**	0.446	**<0.001**	0.478	**<0.001**
Total FFM	0.731	**<0.001**	0.76	**<0.001**	0.677	**<0.001**
Trunk FFM	0.695	**<0.001**	0.78	**<0.001**	0.684	**<0.001**
Limb FFM	0.719	**<0.001**	0.687	**<0.001**	0.633	**<0.001**
Brain volume (cm^3)	0.137	0.211	0.077	0.547	0.36	**0.003**

Significant correlation is indicated as bold.

4.3.3 Contribution of body composition and high metabolic rate organs to ethnic differences in resting energy expenditure

Given that REE differed significantly between Asian-Indians and Chinese, we used linear regression models to investigate whether differences in body composition and HMRO could account for ethnic difference in REE (Table 4.3). In Models 1 to 3, total FFM ($P<0.001$), fat mass ($P=0.008$) and brain volume ($P=0.016$) were significant determinants of REE, independent of ethnicity and explained 54.7% of the variance in REE. Total FFM was the strongest single determinant of REE, which alone accounted for 50.5% of the between-subject variation in REE (data not shown), with relatively minor contributions of ethnicity (1.8%), fat mass (1.1%) and brain volume (1.3%). REE remained significantly lower in Asian-Indians than Chinese (54±22 kcal/day) after adjustment for FFM, fat mass and brain volume. When trunk FFM was added in place of total FFM in Model 4, the difference in REE between Asian-Indians and Chinese was no longer statistically significant (25±22 kcal/day) while using limb FFM ratio in place of total FFM in Model 4 increased the magnitude of difference (85±23 kcal/day) in REE.

Table 4.3 Resting energy expenditure linear regression models

Model and variable	B	SE	Beta	P value	R^2
Model 1: REE = ethnicity + total FFM					0.523
Constant	476.943	71.514		<0.001	
Asian-Indian	-59.065	20.002	-0.149	0.003	
Malay	-15.619	19.629	-0.04	0.427	
Total FFM	21.097	1.329	0.728	<0.001	
Model 2: REE = ethnicity + Total FFM + Fat Mass					0.534
Constant	511.086	72.285		<0.001	
Asian-Indian	-66.96	20.089	-0.169	0.001	
Malay	-22.904	19.683	-0.059	0.246	
Total FFM	19.515	1.477	0.674	<0.001	
Fat mass	3.389	1.438	0.122	0.019	
Model 3: REE = ethnicity + Total FFM + Fat Mass + Brain					0.547
Constant	276.377	122.155		0.025	
Asian-Indian	-53.952	21.505	-0.136	0.013	
Malay	-19.615	20.454	-0.05	0.339	
Total FFM	18.681	1.57	0.645	<0.001	
Fat Mass	4.057	1.516	0.146	0.008	
Brain	224.888	92.422	0.121	0.016	

Model and variable	B	SE	Beta	P value	R^2
Model 4: REE = ethnicity + Trunk FFM + Fat Mass + Brain					
Constant	312.103	123.341		0.012	0.533
Asian-Indian	-25.131	21.744	-0.064	0.249	
Malay	1.141	20.829	0.003	0.956	
Trunk FFM	38.445	3.362	0.631	<0.001	
Fat Mass	3.87	1.56	0.14	0.01	
Brain	227.05	93.955	0.122	0.017	
Model 5: REE = ethnicity + Limb FFM + Fat Mass + Brain					0.52
Constant	346.73	124.185		0.006	
Asian-Indian	-84.8	22.601	-0.214	<0.001	
Malay	-39.507	21.158	-0.101	0.063	
Limb FFM	33.162	3.002	0.611	<0.001	
Fat Mass	5.491	1.517	0.198	<0.001	
Brain	307.11	93.93	0.17	<0.001	

4.3.4 Relations between residual resting energy expenditure and adipokine concentrations

Figure 4.1 shows the relationships between residual REE (difference between measured REE and REE predicted on the basis of FFM and fat mass) and adipokines. Adiponectin trended to correlate positively with residual REE in Chinese (r=0.181, P=0.081) but not Asian-Indians (r=-0.077, P=0543) and Malays (r=0.028, P=0.815). Apelin trended to correlate positively with residual REE in Malays (r=0.219, P=0.066) but not Chinese (r=0.097, P=0.351) and Asian-Indians (r=0.07, P=0.572). MCP1 was positively with residual REE only in Malays (R= 0.303, P=0.021) but not Chinese (R =-0.142, P=0.235) and Asian-Indians (R= 0.157, P=0.28). Higher circulating levels of resistin were associated with greater residual REE only in Asian-Indians (R= 0.275, P= 0.028) but not Chinese (R=-0.04, P=0.714) and Malays(R=-0.189, P=0.114). No significant associations between residual REE with leptin and FGF21 were observed. A significant effect modification by ethnicity was noted for the association between residual REE with MCP1 (P=0.029) and resistin (P=0.045)

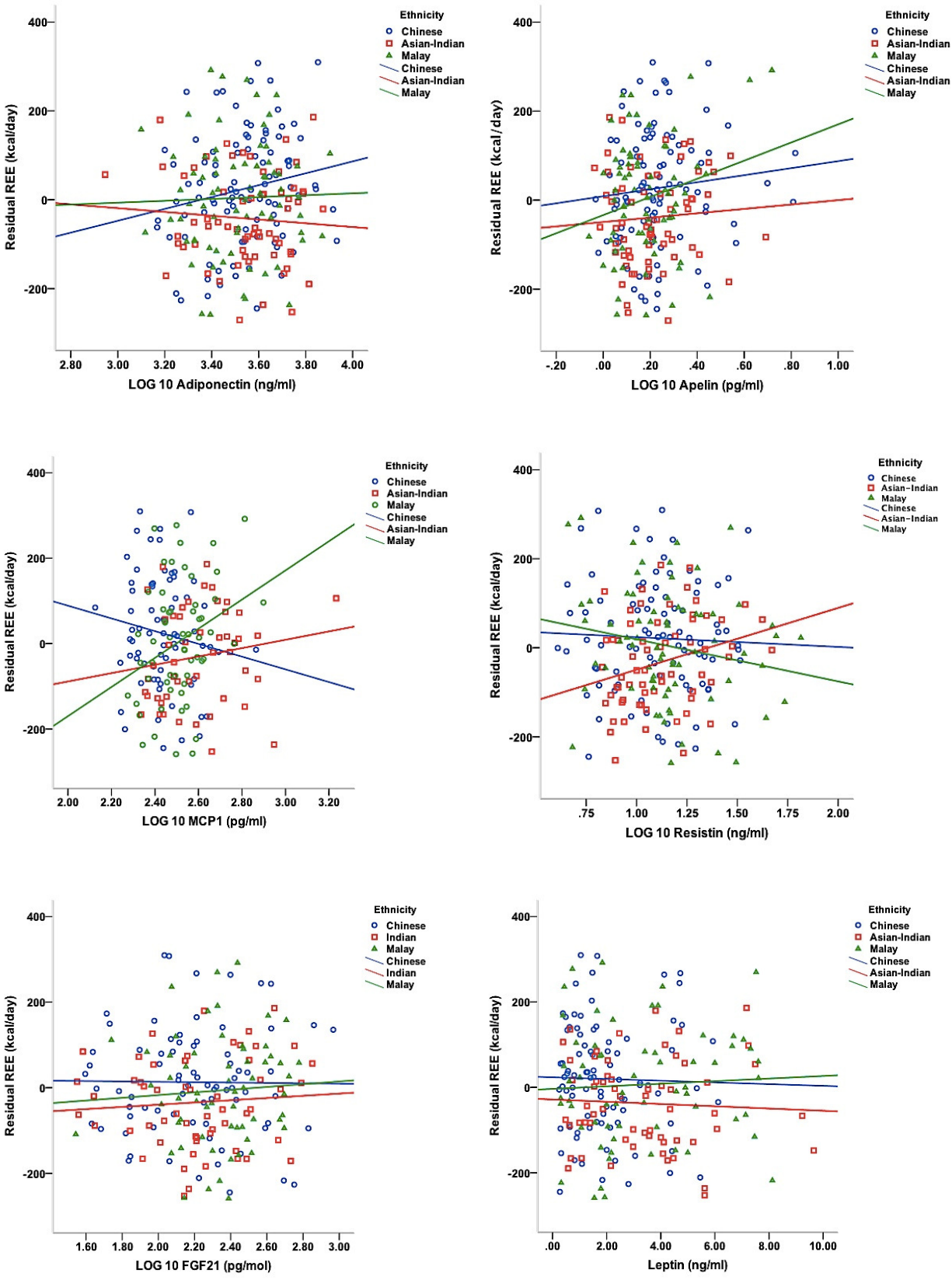


Figure 4.1 Plots of residual resting energy expenditure (difference between resting energy expenditure measured and resting energy expenditure predicted on the basis of fat-free mass and fat mass) against circulating concentrations of adipokines.

4.4 Discussion

In this study, we have shown that, after controlling for body weight, Asian Indians exhibit lower REE than Chinese. This confirms earlier findings in a study of Henry and Rees, showing that the Schofield equation overestimated REE in Chinese, Malay and Indian men by 7.6%, 9.3% 12.8% respectively, suggesting that REE of Chinese is higher compared with their Asian Indian counterparts(131). This ethnic difference could contribute to the excess levels of obesity seen in Asian Indians in Singapore. Even though the difference in REE was relatively small (≈70 kcal/day), over a prolonged period, this degree of caloric excess could give rise to excess body weight of ≈ 2.3 kg per decade in Asian-Indians compared to Chinese, assuming that the energy intake is held constant(197). In contrast, after controlling for body weight, Malays had similar REE to Chinese suggesting that in this ethnic group, the high prevalence of obesity is likely to result largely from excessive energy intake, and not to differences in energy expenditure.

Previous studies that have examine ethnic differences in REE between Chinese, Malays and Asians Indians (131) have relied primarily on body weight, and not body composition, to estimate REE. By studying differences in body composition between ethnic groups, we believe that we may be able to explain these ethnic differences in REE. In all three ethnic groups, we found that FFM, brain volume and fat mass were independent determinants of REE in Chinese, Asian Indian and Malay men. Total FFM was the strongest determinant of REE accounting for half of the variance in REE. It is noteworthy that the contribution of FFM to REE in our study was lower than that reported by Gallagher et al and many authors (2, 24, 198-200) where FFM explained 60-85 % of the variance of REE. It has been argued that the close

relationship between FFM and REE reported by many studies is artificial and attributable to the large heterogeneity of the subject population. If there is a wide range in body weight or FFM in a given population, the contribution of FFM to REE is strong(28). The variation (SD) in FFM in our study (5.9-6.5 kg) was smaller compared to that reported by Gallagher et al (7.1-8.5 kg). Our findings is consistent with the study of Karhunen et al who reported that FFM accounted only 43% of the variation in REE due to the homogenous study group(29). We also found that brain volume was independently associated with REE and explained an additional 1% of the variance in REE over and above that explained by FFM(33). Among the HMRO, brain has one of the highest specific metabolic rates (240 kcal/kg/day) and contributes 23% of total REE even though its weight is only 2% of total FFM(1). Gallagher et al (1)estimated the variation (SD) in brain size of adults to be 0.15 kg in men, which is similar to what we observed in our study population. Therefore, due to the small variation in the brain size, it is not surprising that it does not make a significant contribution to the variation in REE. Another probable reason could be due to the weak association observed between variation in body size and brain mass(1). Despite the low metabolic activity of adipose tissue (4.5 kcal/kg), fat mass was a significant predictor of REE, independent of the effects of FFM, probably due to the wide range in BMI of our study population (18.5 -30.0 kg/m^2). Thus, FFM, FM and brain volume contribute independently, and to a different extent, to REE.

Our data shows that these parameters differ between ethnic groups and may contribute to the ethnic differences in REE. Specifically, although Asian-Indians have greater total FFM compared to Chinese, the trunk FFM and brain volume are lower and limb FFM are higher. This is important because the trunk FFM includes the

high metabolic rate organs (HMRO) including liver, heart, spleen and kidneys. The HMRO (brain, liver, heart, spleen and kidneys) represent only less than 6% of total body weight, but consume approximately 60-70% of the energy expended by fat-free tissue. In contrast, the less metabolically active skeletal muscle, found in the limbs, comprises 40-50% of total body weight while accounting for only 20-30% of REE(1, 27). When we adjusted for trunk FFM, rather than total FFM, the difference in REE between Asian Indians and Chinese is attenuated and is no longer statistically significant. These findings are thus consistent with the hypothesis that Asian Indians have a smaller proportion of their lean tissue as HMROs and a larger proportion as skeletal muscle resulting in a lower REE for any given level of total FFM(22). Previous studies have shown that variation in the organ-tissue proportion of FFM affects ethnic variability in REE independent of the differences in FFM. Hunter et al found that after differences in trunk LBM measured by DEXA were taken into account, the REE difference between premenopausal, nonobese African American and Caucasian women disappeared suggesting that the lower REE in African Americans may be mediated by a lower volume fraction of metabolically active organ mass(22). Gallagher et al confirmed the results of Hunter et al by showing that racial differences in REE were no longer significant after accounting for masses of high metabolic rate organs (liver, kidney, spleen, heart) which were measured by MRI(33).

Next, we examined the roles of adipokines on REE, independent of the effects of FFM and fat mass. We observed significant links between residual REE with MCP1 in Malays and resistin in Asian-Indians. The present study shows high MCP1 and resistin in subjects with high REE. To our knowledge, the physiologic roles of MCP1 and resistin in energy expenditure in humans remain largely undetermined. However,

we postulate that MCP1 and resistin may promote chronic low-grade inflammation through pro-inflammatory cytokines, increasing energy expenditure in a regulatory-feedback manner to prevent fat accumulation in obesity(63-67). MCP1 is a key molecule that mediates infiltration of adipose tissue macrophages (ATM), the dominant source of production of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF-α), interleukin-6 (IL-6) and plasminogen activator inhibitor-1 (PAI-1) during adipose tissue expansion(68). Resistin has been linked to chronic low-grade inflammation as it is homologous to FIZZ1 (found in inflammatory zone 1) and primarily expressed and secreted from monocytes in humans(201, 202). In obese adolescents (203) and adults with obesity and T2DM(204, 205), plasma resistin correlated positively with pro-inflammatory factors. Recently, Lee et al demonstrated that human resistin directly binds to adenylyl cyclase associated protein 1 (CAP1) in monocytes and upregulates cyclic AMP (cAMP) concentration, protein kinase A (PKA) activity, and NF-k B-related transcription of inflammatory cytokines(206). These pro-inflammatory cytokines may regulate energy balance by acting in the hypothalamus of central nervous system(207, 208) and activating thermogenic transcriptional co-activator PGC1-α through phosphorylation by p38 MAP kinase in peripheral tissues(209). Tang et al demonstrated that aP2-p65 transgenic mice with adipose tissue-specific overexpression of the NF-kB p65 subunit exhibited elevated expression of pro-inflammatory genes (MCP-1, TNF-α, IL-6) and were protected from HFD induced obesity and insulin resistance by increased energy expenditure(64). Jiao et al reported that adipose tissue specific IKKβ-overexpressing mice exhibited an increase in energy expenditure and resistance to diet induced obesity(66).

We also observed a trend of higher residual REE with higher apelin levels in Malays. To our knowledge, this is the first study to explore the relationship between apelin and REE in humans. The mechanisms underlying this observation could be mediated by upregulation of brown adipose tissue uncoupling protein 1 (UCP1) activity(60), stimulation of AMP-activated protein kinase (AMPK)-dependent pathway(61) or increased mitochondrial biogenesis in skeletal muscle(62). A trend of positive association between residual REE with adiponectin was also seen in Chinese. Our results support the study of Kim et al who reported positive association between serum adiponectin and REE adjusted for body weight in overweight and obese adolescents(6). Other studies(49, 50) have shown negative associations between total adiponectin and REE even after adjustment for body composition while others have failed to replicate this result(51, 52).(210)

In our study, leptin was not associated with residual REE once the effects of FFM and fat mass had been removed. This means that if an individual had a high concentration of circulating leptin, this had no effect on whether they have high or low REE for their FFM and fat mass. This is consistent with other studies that reported no associations between leptin levels and REE(47, 48). Reports have shown that humans lacking functional leptin production do not have a significantly altered REE(211). Treatment of long-acting pegylated human recombinant leptin in obese men for 12 weeks also produced no effect on weight loss or energy expenditure(211). In contrast, studies in rodents have shown the potential role of leptin in energy metabolism. Administration of recombinant leptin in genetically obese and diet-induced obese mice induces weight loss, by decreasing food intake and increasing resting and physical energy expenditures(8). The discrepancies in the effects of leptin between

human and rodents could be because laboratory rodents are routinely kept in temperatures below the thermoneutral zone. The effects of leptin on REE of mice may reflect the influence of brown adipose tissue thermogenesis. In adults, REE is measured at thermoneutral temperatures and brown adipose tissue levels are too low to be of functional significance for thermoregulation. We also did not find any significant association between FGF21 and the residual variation in REE. Lee et al recently reported that in humans, mild cold-induced increase in FGF21 was associated with cold induced thermogenesis independent of age, gender, fat mass, and lean mass(55). They went on further to show that FGF21 treatment enhanced brown fat thermogenesis in human adipocytes in a depot-specific manner(56). These suggest that FGF21 may be involved only in adaptive thermogenesis but not REE. Overall, ethnicity seem to modulate the relationships between residual REE and adipokines. Reasons for ethnic differences in adipokine regulation of REE are unclear at the moment.

There are limitations in our study. Subjects were not selected from a larger representative population sample and included only young healthy men who have low risk of diabetes or cardio-metabolic diseases. However, this is the age range where the greatest weight gain occurs in our population (189)and thus represents a highly relevant population for the prevention of obesity. Furthermore, excluding those with established diabetes or other diseases avoids the potential confounding or bias that could result from the treatment of these conditions. Nonetheless, we recognize that our study did not include women, and if anything, Malay and Asian Indian women in Singapore are more affected by obesity than men(189). It will be important to extend the findings in this study to women taking into account variation in REE that occurs

in different phases of the menstrual cycle. Despite the advantage of using DEXA to study body composition, this methodology is not without limitations. Trunk FFM and limb FFM are only crude surrogates of HMRO and muscle mass respectively. DEXA is incapable of distinguishing among the various organs and tissues that make up trunk FFM. Studies that have used trunk FFM measured by DEXA were subsequently validated using more sophisticated techniques such as MRI and this gives us confidence that trunk FFM really does reflect the mass of HMRO. However, we recognize that a more precise quantification of these organs man explain a greater proportion of REE. Furthermore, we only assessed the mass/volume of HMRO. Ethnic differences may exist in the specific metabolic rates of organs and tissues that could explain the differences in REE better than mass/volume. However, this has yet to be investigated. A disadvantage of cross-sectional nature of this study is that it does not definitively establish causal or temporal relationships, and should be considered hypothesis generating only.

In conclusion, we have found that Asian Indian men exhibit lower REE than Chinese men for the same body weight. These differences may contribute to the higher prevalence of obesity in this ethnic group. Lower REE in Asian-Indian men may be mediated by smaller size of high metabolic rate organs in the trunk and the brain. To prevent obesity in Asian-Indians, the recommended dietary allowance for energy intake should take into account the lower REE in this ethnic group, and not only on age and body weight as currently recommended by the WHO. In addition, adiponectin, apelin, MCP1 and resistin may be important physiologic candidates that may explain inter-individual variability in REE in different ethnic groups. Consequently, future investigations examining the role of adipokines in the regulation

of energy metabolism and obesity should be ethnicity specific.

Chapter 5 : Metabolic Flexibility Differs among Asian Ethnic Groups in Healthy Lean but not in Overweight or Obese Individuals

5.1 Introduction

Metabolic flexibility (MF) is defined as the capacity of the organism to match fuel oxidation to fuel availability(130). In lean insulin-sensitive individuals, skeletal muscle displays MF with the capacity to increase fat oxidation during fasting and to readily switch from fat oxidation to carbohydrate (CHO) oxidation under insulin stimulation (e.g. during postabsorptive conditions) (212, 213). Conversely, the inflexible skeletal muscle of obese insulin-resistant or type 2 diabetic (T2D) individuals has impaired ability to readily switch from fat oxidation to CHO oxidation in response to insulin(132, 214). Compared with insulin-sensitive individuals, obese insulin-resistant and T2D individuals show higher fasting RQ (132, 214) and blunted increase in RQ during a hyperinsulinemic euglycemic clamp (HEC) (134, 212).

Obesity and insulin resistance are highly correlated. It remains unclear whether the associations between obesity and insulin resistance are independently correlated with MF. Furthermore, MF may differ between ethnic groups. African American men have higher fasting and 24-h RQ than Caucasian men(21). Impaired substrate switching in African American compared to Caucasian women during a high fat diet challenge, two-step pancreatic euglycemic clamp and epinephrine infusion is also observed, which could partly explain the greater prevalence of obesity and insulin resistance in African Americans(140). Among Asian ethnicities, Chinese, Malays and Asian-Indians have different propensity to develop insulin resistance, obesity and T2D. However, MF has not been examined in these ethnic groups. This may be particularly

important given that these, and related, populations, represent up to 2/3 of the world's population.

In this study, we examined the relationship of MF with ethnicity, obesity and insulin resistance. We further sought to understand any associations observed could be explained by a) plasma metabolic intermediates (acylcarnitines, organic acids and amino acids) using metabolic profiling, and b) plasma levels of adipokines such as leptin and adiponectin which have been shown to modulate skeletal muscle substrate utilizations (46, 215, 216).

5.2 Materials and Methods

5.2.1 Subjects

We studied 219 healthy adult males of three of the major ethnic groups (90 Chinese, 65 South Asians and 64 Malays) living in Singapore**.** Details of the subject's selected were previously described in section 2.2.

5.2.2 Anthropometry and body composition measurement

Body weight and height were measured as previously described in Section 2.3. Body composition was measured by dual-energy x-ray absorptiometry (DEXA; Hologic Discovery Wi) as previously described in Section 2.3.

5.2.3 Measurement of respiratory quotient

Indirect calorimetry was performed in the early morning after a 10-hour overnight fast. Subjects were also asked to refrain from intensive physical activity 24 h prior to measurement. Measurements of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were performed by open-circuit indirect calorimetry using a ventilated hood system (Quark CPET, COSMED, Italy) for 1-hour in the fasting condition as previously described. Measurements of VO_2 and VCO_2 were continuously measured for a further 3 hours following a mixed-meal tolerance test (MMTT). The MMTT was a standardized liquid meal (Ensure Plus®, Abbott Nutrition, Abbott Laboratories, Columbus, OH) with a total calorie content of 600kcal which comprised 55% from carbohydrates, 30% from fat (10% saturated fats, 10% polyunsaturated fats, 10% mono-unsaturated fat), and 15% from protein. RQ was calculated from VO_2 and VCO_2 as follows: $RQ=VCO_2/VO_2$. RQ incremental area under the curve [RQ (iAUC)] = total AUC/min – fasting value) was utilized as a measure of MF(136).

5.2.4 Measurement of adipose depots

Liver fat content and intramyocellular lipids (IMCL) were determined using 1H MRS using a tesla MR scanner (Tim Trio, Siemens) as previously described in Section 2.5. Total visceral (VAT) and subcutaneous (SAT) abdominal adipose tissue volumes were measured by magnetic resonance imaging (MRI) as previously described in Section 2.6.

5.2.5 Hyperinsulinemic-euglycemic clamp

Insulin sensitivity was assessed after a 10-hour overnight fast using the hyperinsulinemic euglycemic clamp technique as previously described in Section 2.8. Insulin was infused at a fixed rate of 40 mU/m2 body surface area/minute for 120 min during the clamp, and an infusion of 20% glucose was adjusted to maintain a constant blood glucose of 90 mg/dL. Insulin sensitivity index (ISI) was calculated using the mean glucose infusion rate and steady state insulin concentrations during the final 30min of the clamp.

5.2.6 Enzyme-linked immunosorbent assay

Venous blood samples were drawn from all subjects in the morning after a 10-hour overnight fast. Serum and plasma samples were frozen and stored at -80 °C until tests were performed. Serum leptin and adiponectin were measured by enzyme-linked immunosorbent assay (ELISA) (Millipore, Billerica, MA) as previously described in Section 2.10. Serum leptin was measured by ELISA kit (Millipore, Billerica, MA) with intra-and inter-assay coefficients of variation (CV) of 3.3% and 9.4% respectively. Plasma adiponectin was measured by ELISA kit (Alpco Diagnostics, Salem). The intra-and inter-assay CVs for adiponectin were 3.3% and 6.5% respectively.

5.2.7 Metabolomic Profiling of Organic Acids, Acylcarnitines and Amino acids

Plasma and urine samples were obtained after an overnight fast and 4 h after MMTT. Samples were prepared and stored at -80 °C for later analysis. Acylcarnitines and amino acids were analysed in plasma by tandem MS (MS/MS), and urine organic acids by GC/ MS(170). All MS analyses employed stable-isotope dilution with internal standards from Isotec (St Louis, MO, USA), Cambridge Isotope Laboratories (Andover, MA, USA) and CDN Isotopes (Pointe-Claire, QC, Canada Samples)

5.2.8 Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics for Mac Version 20.0; Armonk, NY: IBM Corp(196). All statistical tests were two-sided, and a P value<0.05 was considered statistically significant. All values were given as means ± SD. All variables were checked for normal distribution, and variables with skewed distributions were log transformed to improve normality. Pearson's correlation analysis was used to assess the relationships between RQ (iAUC) and continuous variables with adjustment for age. The independent t-test was used for two-group comparisons of continuous variables, and ANOVA was used for comparisons in more than two groups, with post hoc Tukey test for pairwise comparisons. ANCOVA was performed to adjust for covariates. To examine whether ethnicity influence the relationship between RQ (iAUC) and measures of adiposity and RQ (iAUC) and insulin sensitivity, we used ANCOVA models with an interaction term "ethnicity X adiposity" included as an independent variable. Repeated-measures ANOVA were used to assess the changes in RQ during MMTT by ethnicity, obesity status and time. The Greenhouse-Geisser adjustment was used when the sphericity assumptions were not fulfilled. Principal components analysis (PCA) was used to reduce a large number

of correlated variables into uncorrelated factors that account for the variance in the dependent variable. The fasting-to-postprandial difference (FPPD) (postprandial – fasting concentration) was calculated for each metabolite. PCA was performed using varimax rotation on the change scores of all measured metabolites and hormones without prior knowledge of ethnicity. Factors with an eigen value ≥2.0 were identified and metabolites with a factor load ≥0.4 were reported as composing a factor. The level of each factor was compared among ethnic groups and between lean and overweight/obese using ANCOVA.

5.3 Results

5.3.1 Subject characteristics

Table 5.1 shows the demographic and clinical characteristics of study participants. Asian-Indians were younger, had greater BMI, %body fat and SAT, but lower VAT and VSR compared with Chinese. IMCL was higher among Asian-Indians than Chinese, but hepatic fat was not significantly associated with ethnicity. There were no significant differences between ethnic groups for fasting NEFA and glucose but fasting insulin level was higher in Asian-Indians than Chinese. Fasting leptin concentrations were higher in Chinese than Malays and Asian-Indians. However, no significant differences between ethnicities were observed for fasting adiponectin concentration. These differences persisted even after adjustment for age.

Table 5.1 Demographic and clinical characteristics of study participants by ethnicity

	Chinese (N=90)		Asian-Indian (N=65)		Malay (N= 64)			
	Mean	SD	Mean	SD	Mean	SD	P value [a]	P value [b]
Age (y)	28.14	5.82	25.74	4.67	27.13	4.96	**0.02**	
Weight (kg)	69.58	10.23	75.00	10.84	73.20	11.49	**0.01**	0.00
Height (cm)	172.01	5.72	173.68	5.76	171.37	6.31	0.07	0.08
BMI (kg/m2)	23.47	2.86	24.85	3.20	24.92	3.65	**0.01**	**<0.001**
Total body fat %	21.06	5.47	24.18	5.95	23.19	6.88	**0.01**	**<0.001**
VAT (L)	964.03	599.18	1158.99	745.24	1077.64	694.81	0.49	**0.02**
SAT (L)	2018.27	950.68	2794.23	1394.13	2622.51	1428.77	**<0.001**	**<0.001**
VSR	0.49	0.21	0.41	0.18	0.43	0.17	**0.04**	0.19
IMCL	9.25	4.08	12.94	6.22	10.23	4.91	**<0.001**	**<0.001**
Hepatic fat (%)	9.07	8.17	10.33	9.86	9.60	9.45	0.89	0.40
ISI	11.12	5.21	8.34	3.77	10.46	5.78	**<0.001**	**<0.001**
NEFA	0.45	0.20	0.51	0.24	0.47	0.21	0.23	0.42
Insulin (mU/L)	11.03	9.90	15.99	23.01	11.08	7.12	**0.03**	**<0.001**
Glucose (mmol/L)	4.53	0.43	4.47	0.36	4.51	0.29	0.66	0.92
Leptin (ng/mL)	2.00	1.49	3.35	2.28	3.25	2.27	**<0.001**	**<0.001**
Adiponectin (ng/ml)	3658.30	1446.29	3867.33	1476.15	3421.85	1474.77	0.23	0.33

[a] P value for comparison between ethnic groups

[b] P balue for comparison between ethnic groups adjusted for age

5.3.2 Relationships between RQ (iAUC) with age and metabolic parameters

Table 5.2 shows the relationship between RQ (iAUC) and a number of physiologic variables including measures of age, adiposity, insulin sensitivity. Age was significantly associated with RQ (iAUC) in Asian Indians and Malays and therefore, other all analyses were adjusted for age where applicable. Overall, RQ (iAUC) negatively correlated with measures of adiposity (BMI, % body fat, SAT and VAT) across all ethnic groups. These relationships remained statistically significance with further adjustment for ISI (data not shown). Liver fat content was also negatively correlated with RQ (iAUC) but this reached statistical significance only in Asian-Indians and Malays. After adjustment for BMI, the associations between RQ (iAUC) with VAT, SAT and liver fat were no longer statistically significant. RQ (iAUC) was positively correlated with ISI among Malays only but this relationship disappeared when adjusted for BMI

Since the correlation coefficient between RQ (iAUC) and measures of adiposity were generally smaller in Chinese than in Asian Indians and Malays, we next examined the interaction between adiposity X ethnicity in relation to RA(iAUC). Figure 5.1 shows the effect of ethnicity on the relationship between RQ (iAUC), adiposity and insulin sensitivity. Effect modification by ethnicity was noted for the association between RQ (iAUC) and measures of adiposity which reached statistical significance for BMI (P=0.051), %body fat (P=0.046) and VAT (P=0.028) between Malays and Chinese. At lower BMI, %body fat, VAT, hepatic fat content and IMCL, Malays and Asian-Indians had significantly higher RQ (iAUC) than Chinese. However at greater BMI, %body fat, SAT, VAT, hepatic fat content and IMCL, the RQ (iAUC) was no longer

different among the ethnic groups or became lower among Malays and Asian-Indians than Chinese.

Table 5.2 Pearson correlation coefficients between RQ (iAUC) with age, adiposity, insulin sensitivity and fasting concentrations of adipokines by ethnicity.

	Chinese			Asian-Indian			Malay		
		P value adjusted for			P value adjusted for			P value adjusted for	
	R	Age	Age and BMI	R	Age	Age and BMI	R	Age	Age and BMI
Age	-0.031	0.772		-0.451	**<0.001**		-0.35	**0.005**	
BMI	-0.244	**0.023**		-0.392	**0.001**		-0.559	**<0.001**	
Body fat %	-0.244	**0.023**		-0.439	**<0.001**		-0.518	**<0.001**	
VAT	-0.252	**0.027**	0.387	-0.3	**0.021**	0.431	-0.481	**<0.001**	0.308
SAT	-0.271	**0.017**	0.291	-0.388	**0.002**	0.967	-0.493	**<0.001**	0.819
IMCL	0.161	0.176	0.07	-0.205	0.125	0.962	-0.152	0.259	0.352
Liver Fat	-0.157	0.194	0.908	-0.365	**0.004**	0.239	-0.354	**0.008**	0.993
ISI	-0.033	0.762	0.084	0.167	0.196	0.476	0.386	**0.002**	0.517
Leptin	-0.219	0.056		-0.352	**0.007**		-0.384	**0.004**	
Adiponectin	0.049	0.657		0.003	0.981		0.122	0.344	

Significant correlation is indicated as bold

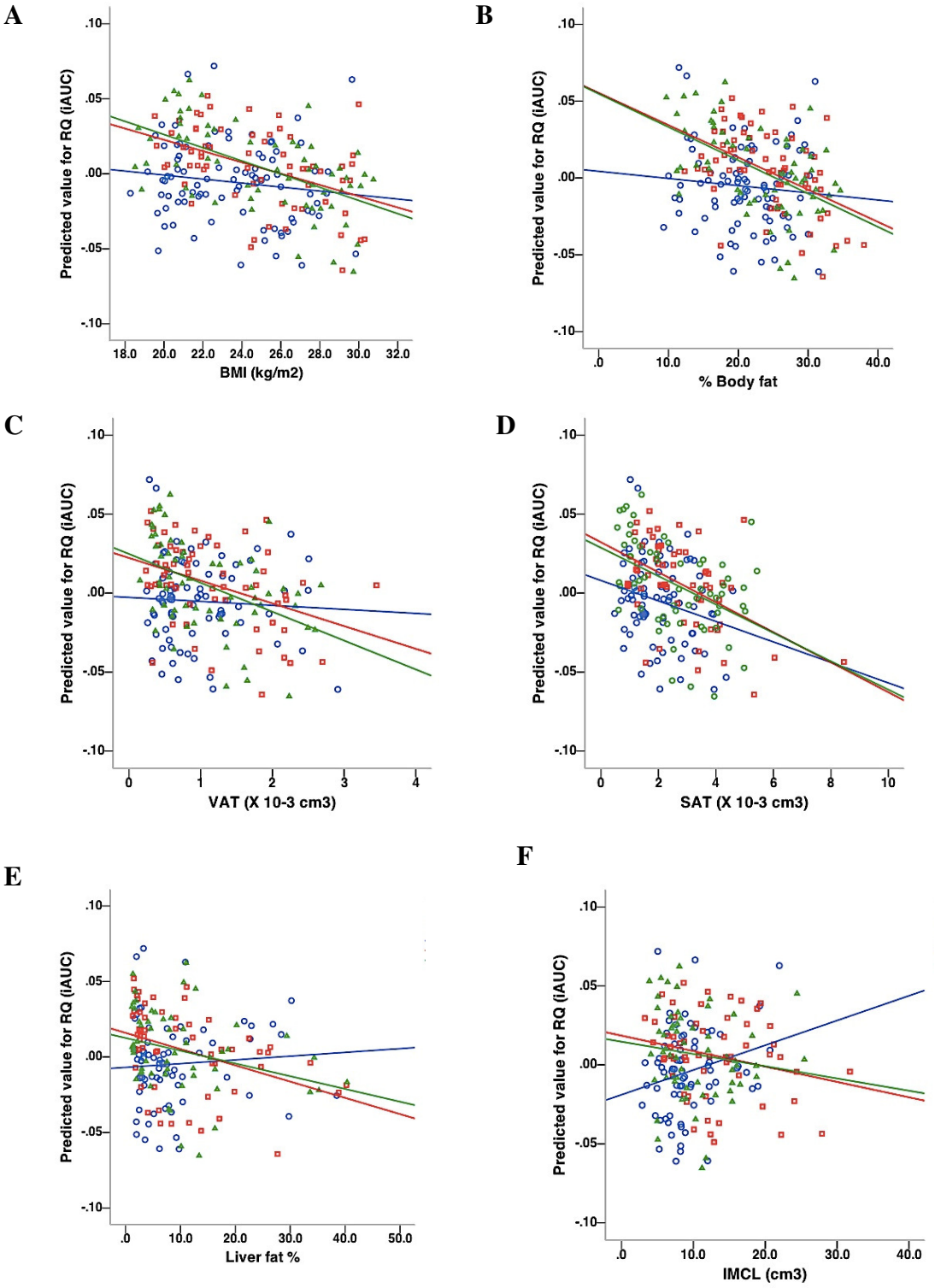


Figure 5.1 Age-adjusted fitted regression plots of RQ (iAUC) against BMI, % body fat, visceral adipose tissue (VAT, cm3), subcutaneous adipose tissue (SAT, cm3), intramyocellular lipids (IMCL) and liver fat (%) for Chinese (blue circle), Asian Indians (red square) and Malays (green triangle). Relationship between RQ (iAUC) and A: BMI (Asian-Indian P interaction= 0.149, Malay P interaction = 0.051). B: % body fat (Asian-Indian P interaction=0.084, Malay P interaction=0.046,). C: VAT (Asian-Indian P interaction =0.122, Malay P interaction=0.028). D: SAT (Asian-Indian P interaction=0.145, Malay P interaction =0.154). E: IMCL (Asian-Indian P interaction =0.012, Malay P interaction=0.016). F: liver fat (Asian-Indian P interaction =0.082, Malay P interaction=0.24).

5.3.3 Impact of ethnicity and obesity on RQ (iAUC)

To better illustrate the associations between obesity, ethnicity and RQ (iAUC), we stratified our data into those who were lean, vs. overweight/obese. Figure 5.2 shows whole body RQ at fasting and after the mixed- meal challenge with time. In the fasting state, the RQ was similar between lean and overweight/obese subjects, and there was no statistically difference between ethnic groups (Figure 5.2 A). The change in RQ following MMTT was statistically significantly (P value for the effect of time <0.001)(Figure 5.2 B). The ability to switch from fat to CHO oxidation, expressed as an increase in RQ (iAUC), was significantly different between ethnic groups (P<0.001), and between lean and overweight/obese subjects (P<0.001)(Figure 5.2 C). Among lean subjects, the RQ (iAUC) was significantly lower in Chinese (0.010 ± 0.004) compared with Asian-Indians (0.035±0.005, P<0.001) or Malays (0.037±0.005, P<0.001). These ethnic differences in RQ (iAUC) between ethnic groups were still statistically significant (P<0.001) after correcting for age and other measures of adiposity (%body fat, VAT, SAT, IMCL and hepatic fat content). Among overweight/obese subjects, there were no ethnic differences observed for RQ (iAUC).

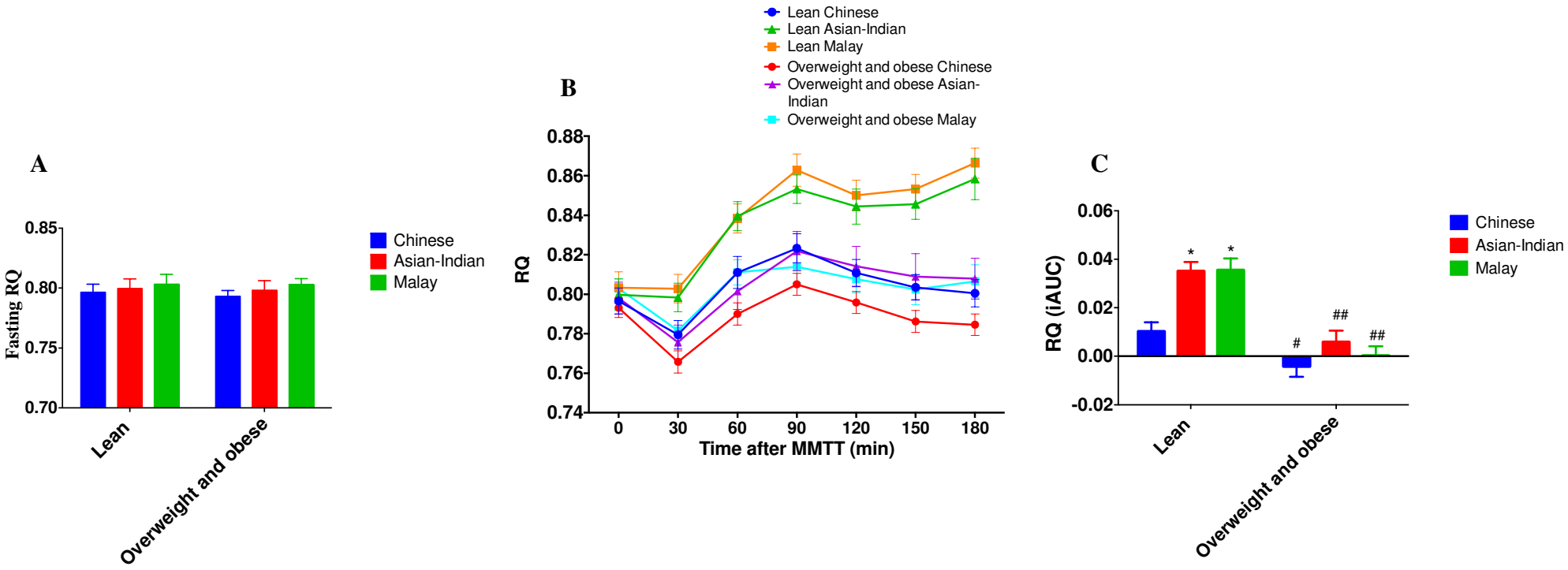


Figure 5.2 (A): Fasting respiratory quotient (RQ). (B): Postprandial RQ after MMTT in Chinese, Asian Indians and Malays stratified into lean and overweight/obese groups. P value for the effect of time was <0.001. (C): RQ (iAUC) defined as incremental postprandial area under the curve. Ethnicity effect was P<0.001, obesity effect was P<0.001. Values represent means ± SD. * P<0.001 compared to lean Chinese, # P<0.05 compared to lean individuals of each ethnic group, ## P <0.001 compared to lean individuals of each ethnic group.

5.3.4 Metabolomic profiling

To gain a more comprehensive snapshot of changes in metabolic fuel selection in the fasted/fed transition, we measured a broad panel of acylcarnitines, amino acids and organic acids that serve as substrates and products of key metabolic pathways at fasting and 4 hours after MMTT. PCA was used to consolidate the fasting to postprandial difference (FFPD) in metabolites into 6 factor components (FC) grouping in biologically plausible clusters (Table 5.3). Ethnic differences in the associations between RQ (iAUC) and FCs were observed (Table 5.4). FC1 which composed of C10, C12:1, C12, C10:1, C14:1, C14:2, C8, C6-DC/C8-OH, C10-OH/C8-DC, C16:1, C16:2, C14 negatively correlated with RQ (iAUC) only in Chinese and Malays but not in Asian-Indians, whereas FC6 which composed of pyruvate and lactate positively correlated with RQ (iAUC) only in Asian-Indians and Malays but not in Chinese. Significant effect modification by ethnicity was noted for the association between RQ (iAUC) and FC6 (P=0.005) but not FC1 (P=0.189). When stratified by BMI, no ethnic differences in FC1 were observed in both lean and overweight/obese individuals (Figure 5.3). FC6 was significantly associated with ethnicity (P=0.015) and obesity category (P<0.001). Among the lean individuals, FC6 was significantly higher in lean Asian-Indians (0.75 ± 0.18, P=<0.001) and Malays (0.34 ± 0.17, P=0.043) compared to Chinese (-0.099 ± 0.13), even after adjusting for age. Among overweight/obese individuals, there were no ethnic differences in FC6. No other FCs were significantly different between ethnicities.

Table 5.3 PCA-derived factor components (FCs)

Factor	Description	Components
1	Medium-chain acylcarnitines	C10, C12:1, C12, C10:1, C14:1, C14:2, C8, C6DC/C8OH, C10OH/C8DC, C16:1, C16:2, C14
2	Amino acids	Phenylalanine, Methionine, Leucine/Isoleucine, Valine, Proline, Tyrosine, Arginine, Glycine, Serine, Alanine, Ornithine
3	Ketone related	Total ketones, β-hydroxybutyrate, C2, C4-OH, non-esterified fatty acids (NEFA),
4	Medium-chain acylcarnitines	C14:1-OH, C14-OH/C12-DC, C20, C18-OH/C16-DC, C4DC/Ci4DC, C12-OH/C10-DC
5	Long-chain acylcarnitines	C18, C16, C18:2, C18:1
6	Glycolysis	Lactate, pyruvate

Table 5.4 Pearson correlation coefficients between RQ (iAUC) with factor components (FCs) by ethnicity, adjusted by age

	Chinese		Asian-Indian		Malay	
	Correlation coefficient	P value	Correlation coefficient	P value	Correlation coefficient	P value
FC1	-0.275	**0.01**	-0.14	0.279	-0.377	**0.003**
FC2	0.098	0.365	0.088	0.497	0.054	0.677
FC3	-0.025	0.819	-0.068	0.601	-0.103	0.424
FC4	-0.04	0.711	0.022	0.867	-0.157	0.223
FC5	-0.166	0.124	-0.116	0.37	-0.015	0.911
FC6	-0.006	0.953	0.385	**0.002**	0.269	**0.034**

Significant correlation is indicated as bold.

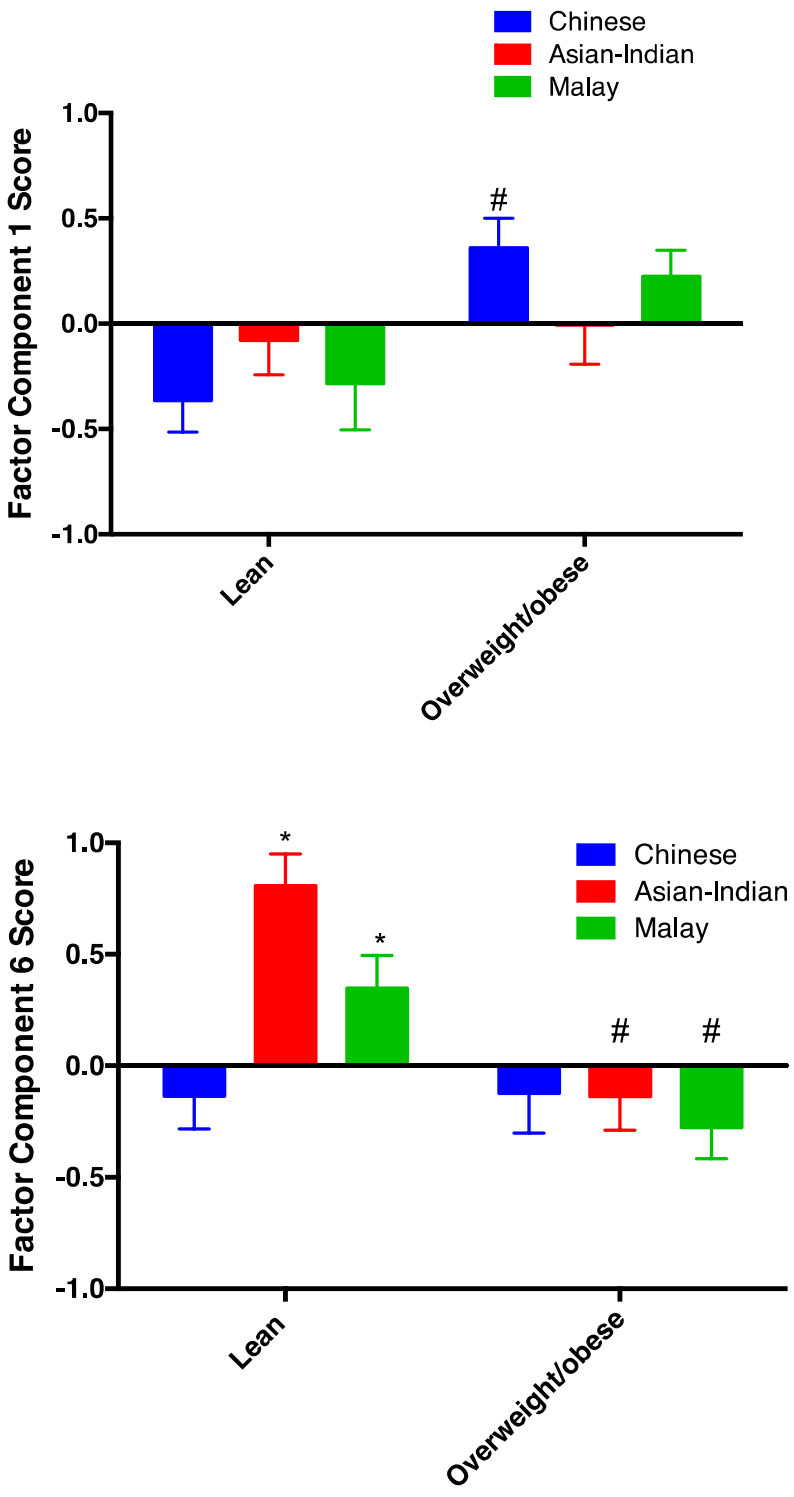


Figure 5.3 PCA derived factor components (FC) in Chinese, Asian Indians and Malays stratified into lean and overweight/obese groups. *P<0.001 compared to lean Chinese, # P<0.05 compared to lean individuals of each ethnic group

5.3.5 Adipokines

Next we wanted to see if adipokines such as leptin and adiponectin could explain the ethnicity interaction with BMI for RQ (iAUC). Leptin has negative correlation with RQ (iAUC) across all ethnic groups, reaching statistical significant in Asian-Indians (r=-0.352, P=0.007) and Malays (r=-0.384, P=0.004) and borderline significance in Chinese (r=-0.219, P=0.056). There were no significant associations between RQ (iAUC) with adiponectin (data not shown). Since leptin correlated significantly with RQ (iAUC), we examined the associations between leptin and BMI. A positive association between leptin and BMI was observed across all ethnic groups and most pronounced in Malays (r=0.756, P<0.001) than Chinese (r=0. 651, P<0.001) and Asian-Indians (r= 0.605, P<0.001). Upon adjustment for leptin, the significant association between RQ (iAUC) and BMI disappeared in Chinese (r= -0.137, P=0.238), Asian-Indians (r=-0.24, P=0.075) and Malays (r= -0.245, P=0.08).

5.4 Discussion

In Asian men, fasting RQ was not associated with either ethnicity or obesity. MF, as measured by RQ (iAUC) was independently associated with obesity but not insulin resistance in Chinese, Asian-Indians and Malays. In Malays, ISI was associated with RQ (iAUC) but the association was completely attenuated by adjustment for obesity. The relationship between MF and obesity is modulated by ethnicity. In lean individuals, Chinese had lower MF than Malays and Indians. However, in overweight/obese individuals, MF was similar between ethnic groups. These ethnic differences seem to relate to differences in the propensity to increase glycolysis, rather than suppression of fat oxidation, after a meal. Thus, altered glycolysis should be considered when examining inter-individual differences in MF.

5.4.1 Fasting RQ

Several studies have demonstrated elevated fasting RQ in skeletal muscle from obese, and type 2 diabetic subjects compared to control subjects(132, 212). In contrast, other studies have reported a positive association between fasting fat oxidation and body fat mass(217-219). Flatt postulated that an increase in fat mass will lead to an increase in fat oxidation in response to a high fat diet, providing a new plateau of weight maintenance at a point where fat oxidation equals fat intake(220). In our study, fasting RQ was not significantly different between lean and overweight/obese individuals, which is consistent with studies showing that fasting RQ was not related to fat mass or percentage body fat(139, 221, 222). Studies that have examined the impact of ethnicity on fasting RQ have yielded conflicting results. Chitwood et al showed a significantly higher RQ in lean African-American women compared to lean white women, which could be a contributory factor to greater obesity in African Americans.

In contrast, no difference in resting RQ between African-Americans and Caucasians was observed in many studies(23, 37, 223). Similarly, in our study, fasting RQ was not different between ethnic groups although a trend towards higher use of carbohydrates as fasting energy substrate was observed in Malays, followed by Asian Indians and Chinese. A point to note is that fasting RQ may not be a reliable assessment of the capacity to switch from CHO to fat oxidation as RQ under fasting conditions is highly sensitive to differences in energy balance and diet composition within the few days preceding the measurement(224, 225). Either positive energy balance or high CHO intake can transiently elevate RQ(226). In addition, resting conditions may not exert a metabolic challenge for muscle mitochondrial to oxidize fat. Therefore, defects in skeletal muscle fat oxidation capacity is unlikely to be observed in resting conditions(227).

5.4.2 Obesity

Numerous studies have shown that obesity is associated with a blunted increase in RQ during a HEC(130, 132, 216, 228) or oral glucose tolerance test (OGTT)(229). The insulin response occurring after MMTT may be affected by enteric hormones, amino acids from foods, gastrointestinal motility, neural impulses during ingestion of foods, and gastric emptying time, besides glucose obtained from CHO. As such, we believe the MMTT represents a more physiological test that the HEC, that is performed at supra-physiological levels of insulin, or the OGTT where only pure glucose is administered for the stimulation of insulin secretion. A few have studied MF using a mixed meal tolerance test (MMTT). Blaak et al showed that postprandial fat oxidation after a high fat meal decreased with increasing BMI(217). Huffman et al demonstrated that three months of caloric restriction significantly increased the fasting to postprandial difference in circulating acylcarnitines and free-fatty acids

after MMTT, indicating greater suppression of fat oxidation(230). In our study, we found that postprandial switch from fat to CHO oxidation was negatively associated with BMI and body fat %, which is in line with the findings of these studies. In contrast, Weyer et al have reported similar MF in obese and lean individuals exposed to high CHO diet(231). However, in this study (Weyer et al), MF was measured as the 24 hour RQ after 48 hours of overfeeding with a high CHO diet. It is not clear if these findings are directly comparable to ours, and those of Blaak et al and Huffman et al, which examine acute changes after a single meal during the fasting to the fed transition.

5.4.3 Insulin resistance

The lack of association between ISI and MF is not surprising. Several studies have reported that metabolic inflexibility in T2D subjects assessed by HEC is not due to a primary impairment in glucose oxidation in muscle, but can be explained fully by impaired glucose uptake(134, 214, 232). In one of these studies, they reported that metabolically inflexible subjects had similar metabolic characteristics but higher BMI compared with the flexible subjects(134), which is very much in line with our finding that obesity rather than insulin sensitivity is a determinant of MF. Kelley and Mandarino showed that insulin stimulated leg glucose oxidation was not significantly different between T2D subjects and controls after matching leg glucose uptake(214). Galgani et al also reported that the lower ΔRQ observed in T2D versus nondiabetic individuals, during hyperinsulinemic conditions was no longer significant after adjusting for glucose disposal rate.

5.4.4 Ethnicity

The relationship between MF and obesity is modulated by ethnicity. A larger RQ (iAUC) was observed amongst lean Asian Indians and Malays compared with Chinese indicating a greater propensity to oxidize glucose, relative to fat, in these ethnic groups during the fasting to fed transition. In overweight and obese individuals, the differences in RQ (iAUC) between ethnic groups were no longer seen. The metabolomics profile provides some insight regarding these findings. Our data supports the hypothesis that the determinants of MF may differ in different ethnic groups. In Chinese, increase in MF primarily correlates with the decrease in even chain acylcarnitines, which indicates a reduction in fatty acid oxidation, during the fasting to fed transition. In Indians, MF is associated with higher levels of pyruvate and lactate. Higher pyruvate levels may enhance flow of glucose derived pyruvate carbon into the TCA cycle via pyruvate dehydrogenase, leading to increase RQ. Thus, we interpret this as an increase in glycolysis and glucose oxidation leading to an increase in RQ. In Malays, changes in both fatty acid and glucose oxidation are implicated in MF. In response to MMTT, a greater increase in pyruvate and lactate, were seen in lean Asian-Indians and Malays compared to Chinese.

We also explored the possibility that adipokine secretion may differ between individuals from different ethnic groups with increasing obesity and that may underlie the associations observed. We found a negative correlation between leptin and MF, which is supported by the study of Hwa et al, showing that leptin administration in ob/ob mice reduces RQ, an indication of a reduction in relative carbohydrate oxidation rate and of a rise in the fat oxidation rate(233). Human studies have shown that leptin stimulates skeletal muscle fatty acid oxidation through the 5'-AMP-activated protein kinase (AMPK) pathway(234). Wauters et al demonstrated that polymorphisms in the leptin receptor gene are associated with relative fat oxidation

rates in response to a glucose load(235). Furthermore, adjustment for leptin attenuated the association between obesity and MF such that the no statistically association between BMI and RQ(iAUC) was observed after this adjustment. This suggests that a large portion of the association between obesity and MF could potentially be mediated by the actions of leptin.

Previous studies have reported ethnic differences in the associations between leptin and obesity(236, 237). Moriomoto et al recently showed that higher levels of leptin with greater BMI was most prominent in African American, followed by White, Latino, Native Hawaiians and Japanese American women(236). In our study, Chinese show less of an increase in leptin with increasing obesity compared to Malays, with the interaction reaching statistical significance for %body fat. Also, the magnitude of the association between leptin and MF is also smaller in Chinese compared to Asian-Indians and Malays although this did not reach statistical significance. Thus, with increasing obesity, the increase in plasma leptin is less in Chinese than in Malays, and this may be the reason that the association between obesity and MF is less pronounced in Chinese than in Malays.

In our study, adiponectin was not associated with MF, which opposes previous findings(216, 238). Sparks et al reported higher fasting adiponectin levels was associated with greater insulin stimulated glucose uptake and oxidation in healthy young males(216). Asterholm also demonstrated adiponectin improved MF in mice exposed to chronic high fat diet feeding by enhancing clearance of circulating fatty acids and mitochondrial density in adipocytes(238).

We considered several possible reasons as to why lean Chinese are less efficient in switching from fat to CHO utilization than their Asian-Indian and Malay counterparts.

Studies have reported regional variations in the lipolytic activity of human adipose tissue where the rate of lipolysis is lowest in the subcutaneous region and highest in the visceral region. The lipolytic β1, β2 and β3 adrenoceptors are most active in the visceral fat cells and antilipolytic insulin receptors, α2 adrenoceptors and adenosine receptors are most active in the subcutaneous fat cells(239). In our study, VSR is higher in lean Chinese than the other ethnic groups, suggesting that the adipose tissue exhibit increased lipolysis and reduced insulin mediated antilipolysis, leading to higher circulating FFA. Higher plasma FFA may increase lipid oxidation and in turn reduce insulin stimulated glucose uptake and oxidation(216, 240). Although we did not find differences in plasma non-esterified fatty acids (NEFA) between ethnic groups during fasting (Chinese (0.48±0.19), Asian-Indians (0.56± 0.26), Malays (0.49± 0.27)) and postprandial conditions (Chinese (0.12±0.058), Asian-Indians (0.12± 0.063), Malays (0.15± 0.091)), differences in NEFA turnover rates or NEFA availability may have been present. Secondly, adipose tissue lipid storage capacity may impact MF. We postulate that Chinese may have a lower capacity for fat storage and is less able to buffer the influx of dietary fat entering the circulating in the postprandial period and prevent excess exposure of peripheral tissues to fatty acids.

5.4.5 Clinical Implications

In lean Asian-Indians and Malays, greater suppression of fat oxidation after a mixed meal creates a metabolic profile favoring fat storage. These individuals may be less able to switch on fat oxidation in response to an increase in dietary fat intake, and they may therefore be prone to store fat as adipose leading to a partitioning of energy toward fat mass at the expense of lean mass. Moreover, previous studies have suggested that greater CHO oxidation and lesser fat oxidation may predispose certain individuals to store less glycogen and therefore experience more hunger. This

increased hunger may lead to ingestion of more total energy leading to chronic positive energy imbalance and gain in body fat (46, 241). Eckel et al reported that individuals with increased CHO balance at the end of a 15-day high-CHO diet gained less body weight and fat mass over the next 4 years than those with lower CHO balance(242). Zurlo et al have also shown high 24-h RQ (high ratio of CHO to fat oxidation) to be a predisposing risk factor for fat mass gain, independent of energy expenditure(7). As such, higher rates of CHO relative to fat oxidation in lean Asian-Indians and Malays compared to lean Chinese, may predispose them to deplete glycogen stores and ingest more total energy. Data from the National Health Survey 2010 supports this and have shown that obesity prevalence is highest in Malays (24.0%) followed by Asian-Indians (16.9%) and Chinese (7.9%). Another implication of lower CHO oxidation in lean Chinese is increased de novo lipogenesis (DNL) of excess dietary CHO in adipose tissue and liver. In our study, lean Chinese exhibit significantly higher hepatic fat content than their Asian-Indian and Malay counterparts, therefore are at a higher risk for developing non-alcoholic fatty liver disease. No differences in hepatic fat between ethnic groups were observed in overweight and obese individuals.

5.4.6 Limitations

Due to cross-sectional comparisons of lean and obese subjects, whether impaired MF is a primary defect or arises secondarily after an individual has become obese cannot be effectively addressed. In addition, the issue of whether lower CHO oxidation observed at a whole body level in lean Chinese compared to Asian-Indians and Malays is the consequence of reduced glucose uptake, impaired CHO oxidative capacity or enhanced ability to store glycogen in skeletal muscle could not be determined in our study. We also examined a very specific population of healthy

young men, hence it is impossible to extrapolate these findings to women, older individuals or those with T2D. In summary, the impaired capacity in switching from fat to CHO oxidation is associated with obesity, increased volumes of VAT, SAT and liver fat, an effect present primarily in Asian-Indians and Malays. Insulin resistance was not associated with MF after adjusting for obesity, supporting the concept that MF is associated with obesity per se. Serum leptin and RBP4 may mediate the interaction between ethnicity, obesity and MF.

In summary, MF, as measured by RQ (iAUC) is associated with obesity per se and not with insulin resistance. Ethnicity modulates the relationship between MF and obesity. In lean individuals, Chinese had lower MF than Malays and Indians. However, in overweight/obese individuals, MF was similar between ethnic groups. This study provides evidence that ethnic differences should be considered when evaluating MF.

Chapter 6 : The role of skeletal muscle metabolism in resting energy expenditure and metabolic flexibility in Chinese and Asian-Indian men

6.1 Introduction

Previously, we have shown that after controlling for body weight, Asian Indians exhibit lower resting energy expenditure (REE) than Chinese (Chapter4). Furthermore, Chinese displayed lower metabolic flexibility (MF) in substrate switching from fat to carbohydrate oxidation in response to a mixed meal than Malays and Indians in lean individuals. In contrast, MF was similar between ethnic groups in overweight/obese individuals (Chapter 5). However, the molecular basis for ethnic variability in REE and MF is not known.

Skeletal muscle is not only a major insulin target tissue responsible for approximately 80% of insulin-responsive glucose uptake, it also involved in glycogen storage, lipid oxidation and nonshivering thermogenesis. A significant proportion of variability in REE after adjustment for fat-free mass (FFM) and fat mass has been shown to be associated with the variability in skeletal muscle metabolism(73). Mitochondrial proton leak, by which the potential energy in the proton motive force is dissipated as heat instead of being trapped in ATP, is postulated to be an important determinant of metabolic rate as it accounts for 20% of the basal oxygen consumption rate in rats(243). However, the physiological role of proton leak in humans is not fully understood. Adenine nucleotide translocase 1(ANT1) and uncoupling protein 3 (UCP3) localized on the inner mitochondrial membrane are reported to mediate basal and inducible proton leak of mitochondria in skeletal muscle, hence are likely

candidates to underlie the physiological variability in REE. Transgenic mice that overexpress human UCP3 in skeletal muscle are hyperphagic but weigh less than their wild-type littermates(86). In human studies, UCP3 mRNA expression was positively correlated with REE adjusted for fat-free mass and fat mass (119).

Given the prominent role of skeletal muscle in substrate selection, muscle mitochondrial function is likely to have a prominent role in MF. However the link between mitochondrial function and MF remains controversial. Myotubes from lean subjects exhibit increased mitochondrial respiration (state 3) in the presence of palmitoyl carnitine compared to obese subjects, indicating that skeletal muscle of obese individuals inherently lacks metabolic flexibility (145). In contrast, studies have also reported that defective insulin-stimulated glucose disposal rate is the main determinant of metabolic inflexibility suggesting that MF is merely a consequence of insulin resistance in obesity and type 2 diabetes (T2D)

The purpose of this study was to investigate potential inter-individual differences in skeletal muscle characteristics that can explain variability in REE and MF and also the impact of ethnicity on molecular factors affecting skeletal muscle energy metabolism. Before answering these questions, we want to establish whether primary human myoblast or myotube is a better in vitro experimental model for the study of metabolism and energy homeostasis.

6.2 Materials and methods

6.2.1 Subjects

We selected myoblasts from 12 Chinese subjects (4 lean and 8 overweight/obese) and 11 Asian-Indian (5 lean and 6 overweight/obese). Subject details are described in Section 2.2.

6.2.2 Anthropometry and body composition measurement

Body weight and height were measured as previously described in Section 2.3. Body composition was measured by dual-energy x-ray absorptiometry (DEXA; Hologic Discovery Wi) as previously described in Section 2.3.

6.2.3 Measurements of resting energy expenditure and respiratory quotient

Open-circuit indirect calorimetry was performed in the early morning after a 10-hour overnight fast. Subjects were also asked to refrain from intensive physical activity 24 h prior to measurement. Measurements of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were performed using a ventilated hood system (Quark CPET, COSMED, Italy) for 1-hour in the fasting condition as previously described. Measurements of VO_2 and VCO_2 were continuously measured for a further 3 hours following a mixed-meal tolerance test (MMTT). RQ was calculated from VO_2 and VCO_2 as follows: $RQ=VCO_2/VO_2$. Whole body MF was calculated as the incremental area under the curve (iAUC) for RQ (136)

6.2.4 Muscle Biopsies

Percutaneous muscle biopsy was obtained from the vastus lateralis under local anaesthesia and processed for myoblast and myotube culture as described in Section 2.11.

6.2.5 Mitochondrial respiration

Mitochondrial respiration was measured in primary human myotubes using a Seahorse XF24 Analyzer (Seahorse Biosciences, North Billerica, MA) as previously described in Section 2.12

6.2.6 Quantification of gene expression

mRNA gene transcripts for selected genes were examined using techniques described in Section 2.13

6.2.7 Western Blot

Quantification of protein abundance were performed using techniques described in Sections 2.14.

6.2.8 Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics for Mac Version 20.0; Armonk, NY: IBM Corp(196). All statistical tests were two-sided, and a P value<0.05 was considered statistically significant. Comparisons of mitochondrial respiration, mRNA and protein expression levels between the lean and overweight and between Chinese and Asian-Indians were conducted using Kruskal-Wallis test. Correlations between REE with mitochondrial respiration and proteins were conduced using Spearman correlation test.

6.3 Results

6.3.1 Anthropometry and clinical characteristics of subjects

Subject characteristics are presented in Table 6.1. Overweight/obese Asian-Indians were younger and heavier than their Chinese counterparts. Similar FFM was observed between ethnic groups, although overweight/obese Asian-Indians had a significantly higher fat mass than Chinese. Fasting plasma NEFA concentrations were higher in overweight/obese Chinese than Asian-Indians. No differences in plasma concentrations of insulin and glucose were noted between ethnicities. REE was higher in Chinese than Asian-Indians, though no statistically significant differences were observed. RQ (iAUC) was significantly greater in Asian-Indians than Chinese in the lean but not in overweight/obese individuals.

Table 6.1 Demographic and clinical characteristics of Chinese and Asian-Indians stratified into lean and overweight/obese groups.

	Lean (N=9)					Overweight and obese (N=14)				
	Chinese (N=4)		(N=5)			Chinese (N=8)		Asian-Indian (N=6)		
	Mean	SD	Mean	SD	P value [a]	Mean	SD	Mean	SD	P value [b]
Age	25.25	5.32	23.00	1.87	0.80	34.25	4.65	26.83	5.53	**0.05**
BMI	20.96	1.09	20.55	1.05	0.33	26.32	1.60	28.75	1.81	**0.02**
Total Fat Mass	9.21	4.29	8.58	2.59	0.81	19.19	5.67	28.10	6.38	**0.01**
Total FFM	52.57	3.76	49.93	2.62	0.22	57.53	5.95	57.93	7.60	0.70
Glucose	4.35	0.21	4.30	0.34	0.62	4.82	0.37	4.39	0.33	0.06
Insulin	6.28	2.74	9.84	10.78	0.90	12.91	6.06	19.42	8.46	0.12
NEFA	0.46	0.31	0.35	0.23	0.72	0.51	0.15	0.32	0.14	**0.04**
REE	1624	226	1463	94	0.33	1706	205	1620	234	0.90
Fasting RQ	0.82	0.07	0.82	0.05	0.87	0.81	0.03	0.82	0.05	0.80
RQ (iAUC)	0.002	0.012	0.028	0.013	**0.01**	-0.019	0.026	-0.017	0.044	0.85

[a] P value for comparison between ethnic groups in lean individuals

[b] P balue for comparison between ethnic groups in overweight and obese individuals

6.3.2 Comparison of metabolic characteristics in primary human myoblast and myotubes

When visualised microscopically, primary human myoblasts were mononucleated (Figure 6.1). The myotubes at day 7 of differentiation appear multi-nucleated, elongated, with a fiber like structure, showing that fusion and differentiation of myoblasts into myotubes has occurred. To verify that primary human myoblast were fully matured into multinucleated myotubes, gene expression of myogenic markers of differentiation were measured in myotubes at days 0, 4, 8 and 12 of differentiation (Figure 6.2). MyoD is a member of the family of myogenic regulatory factor involved in the commitment of myoblast cells to differentiation. MyoD mRNA expression was highest at day 4, followed by a reduction at day 8 of differentiation, which is consistent with studies showing that MyoD is upregulated in the early stages of myogenesis (Figure 6.2A). The final stage of differentiation can be identified by the expression of the structural proteins such as α-sarcomeric actin and myosin heavy chain (MHC) that form part of the contractile apparatus. The expression of myosin heavy chain was shown to be highest at day 8 of differentiation but later decreased at day 12 (Figure 6.2B). Longer durations of differentiation may lead to myotube detachment and subsequent cellular death.

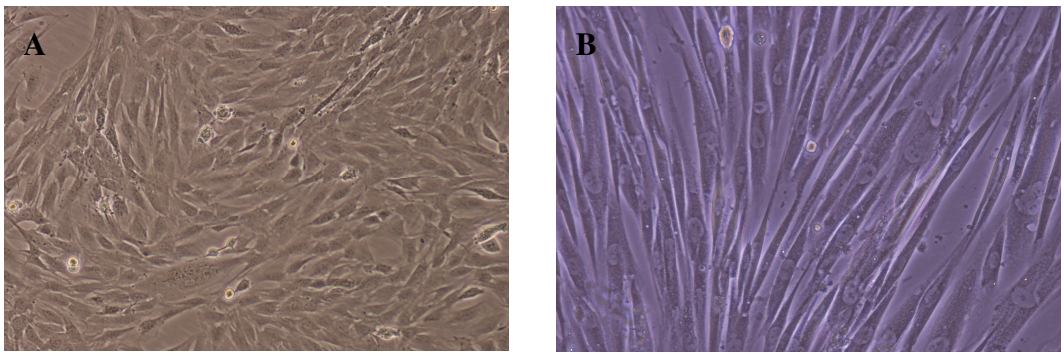


Figure 6.1 Phase contrast appearance of human satellite cell culture. Morphological appearance was investigated by phase contrast microscopy. (A) Mono nucleated myoblast cells under proliferation (10X). (B) Day 7 differentiated multinucleated myotubes (20X)

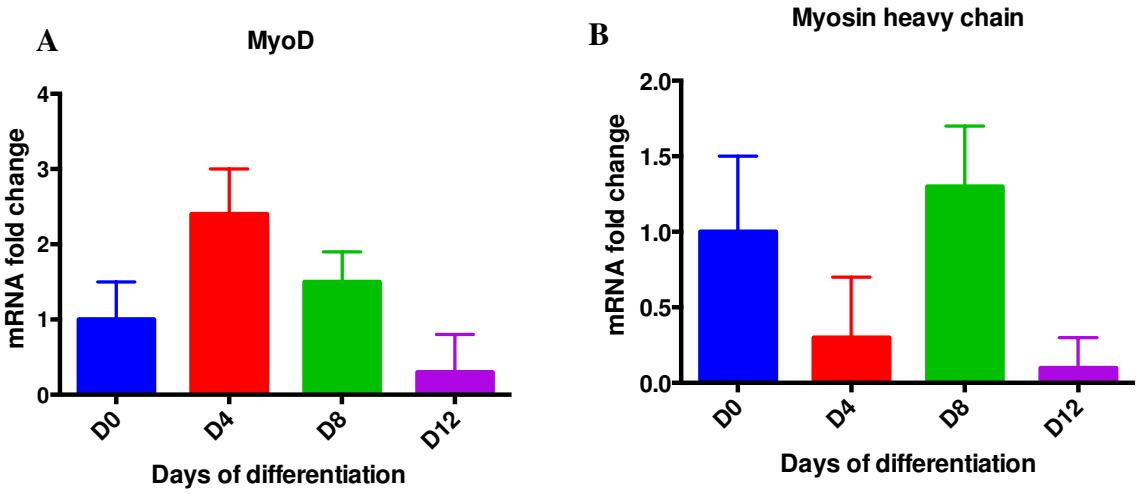


Figure 6.2 Gene expression of myogenic markers of differentiation in primary human myoblast cultures from during day 0, day 4, day 8 and day 12 of differentiation.

High resolution respirometry was also performed in myoblasts and myotubes. In Figure 6.3, both myoblasts and myotube cultures were able to respond maximally to the different compounds added to the assay medium, showing that different parameters of mitochondrial respiration can be measured in both in vitro cell culture models. Next we measured the gene expression of uncoupling proteins UCP2, UCP3 which are major determinants of energy metabolism. UCP2 and UCP3 were upregulated in the myotubes at day 4 and day 8 of differentiation (Figure 6.4). Therefore, myotubes is a better in vitro experimental model for the study of energy metabolism and uncoupling proteins compared to myoblast.

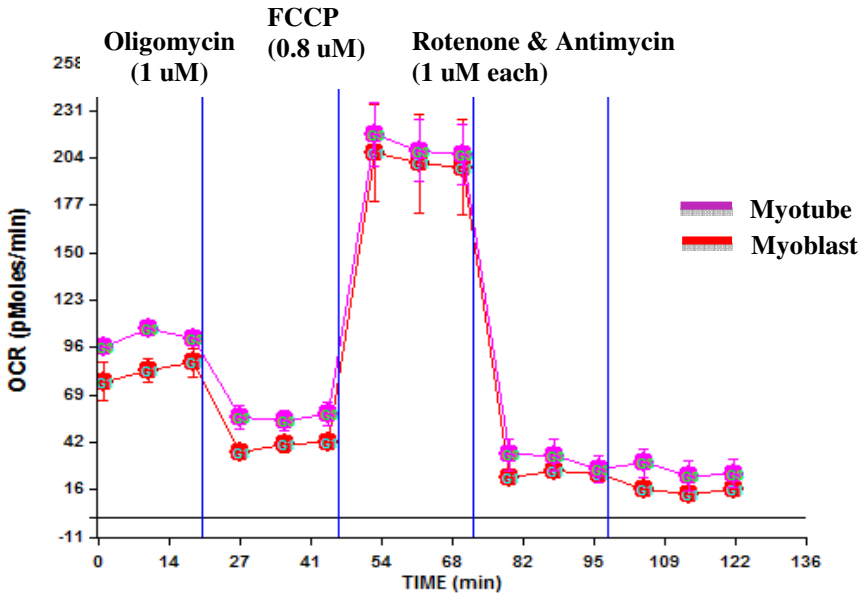


Figure 6.3 Mitochondrial bioenergetic profile in primary human myoblasts and myotube cultures.

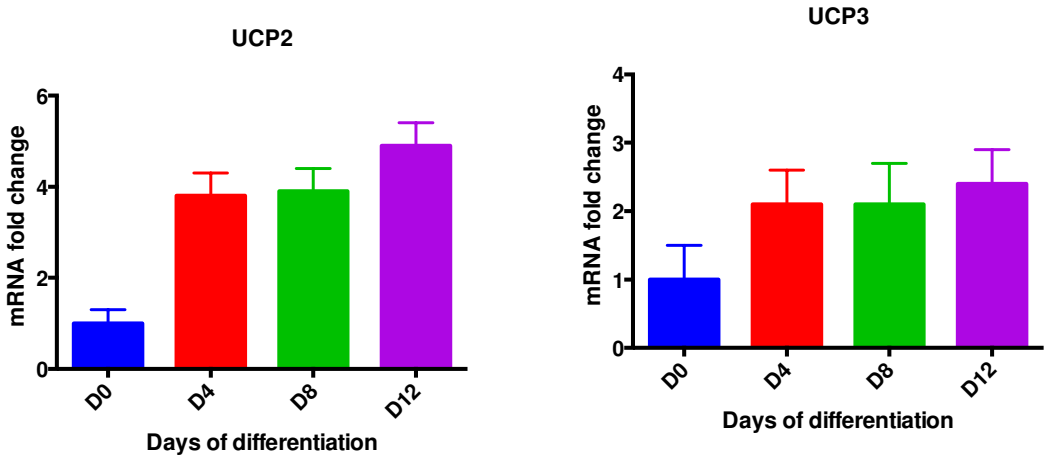


Figure 6.4 Gene expression of uncoupling proteins in primary human myoblast cultures from during day 0, day 4, day 8 and day 12 of differentiation.

6.3.2 Expression of genes in TCA cycle, OXPHOS and uncoupling in Chinese and Asian-Indian myotubes

Expression of oxidative and lipid oxidation genes in primary human myotubes is shown in Figure 6.5. The expression of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC1A) was 2.18 fold greater (P=0.077) in lean Asian-Indians than lean Chinese, and 2.28 fold greater (P=0.02) in overweight/obese Asian-Indians than overweight/obese Chinese. There was no significant effect of obesity or ethnicity on expression of isocitrate dehydrogenase (IDH3G) involved in TCA cycle. NADH dehydrogenase (NDUFB4) and cytochrome c oxidase (COX15) involved in electron transport chain (ETC), ATP synthase (ATP5J2) involved in OXPHOS, carnitine palmitoyltransferase 1B (CPT1B) and HADHA involved in lipid oxidation.

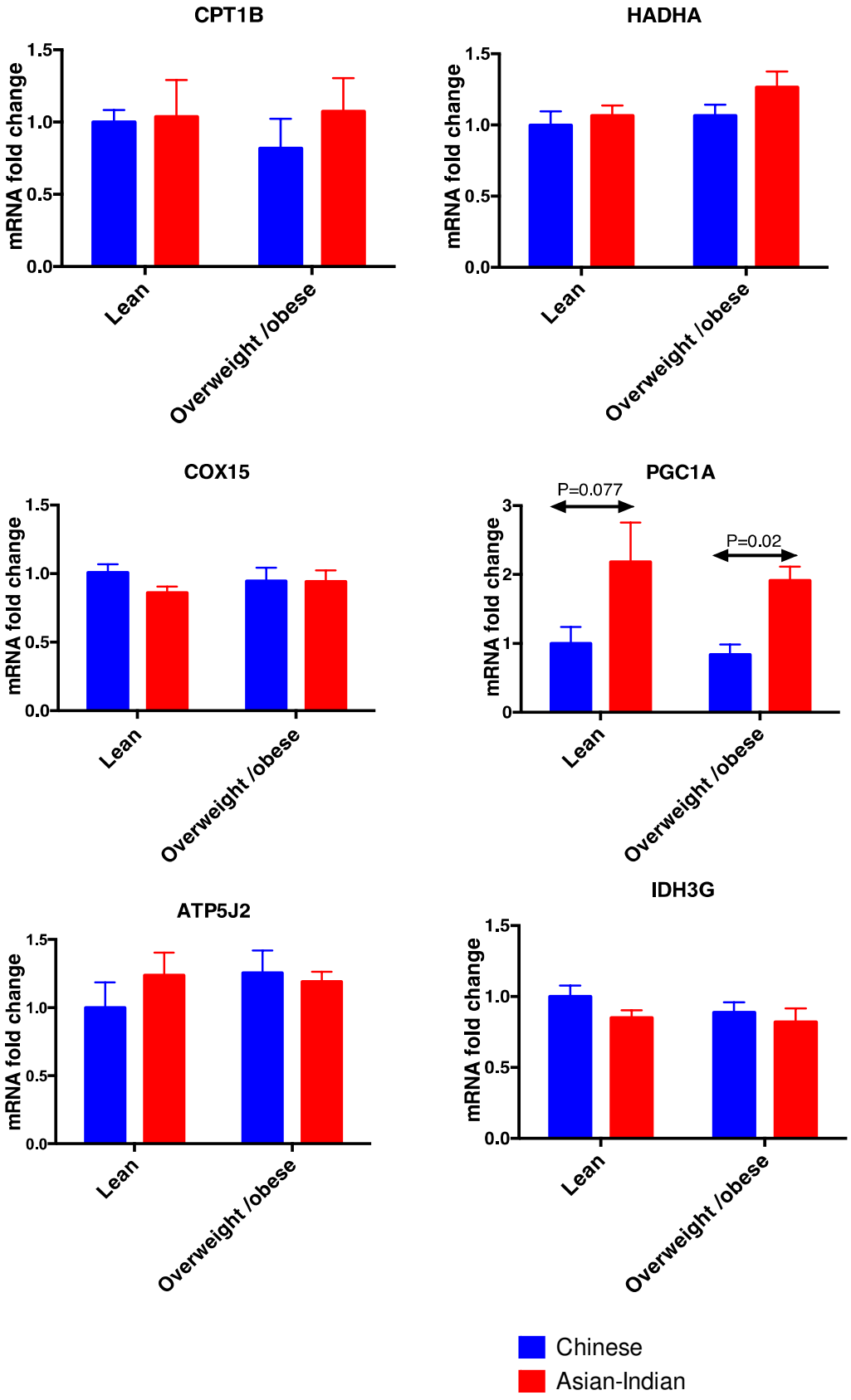


Figure 6.5 Expression of metabolic genes involved in fatty acid oxidation, oxidative phosphorylation and TCA cycle in Chinese and Asian-Indian myotubes, stratified into lean and overweight/obese groups. Data are expressed as mean ±SD.

6.3.3 Protein expression in Chinese and Asian-Indian myotubes

Asian-Indians exhibited significantly higher protein levels of uncoupling protein (UCP3) and adenine nucleotide translocase (ANT1) involved in proton leak and citrate synthase (CS) involved in TCA cycle than Chinese, in both lean and overweight/obese states (Figure 6.6 and 6.7). Protein levels of cytochrome c oxidase (COXIV) were not significantly different between ethnic groups or between lean and overweight/obese individuals.

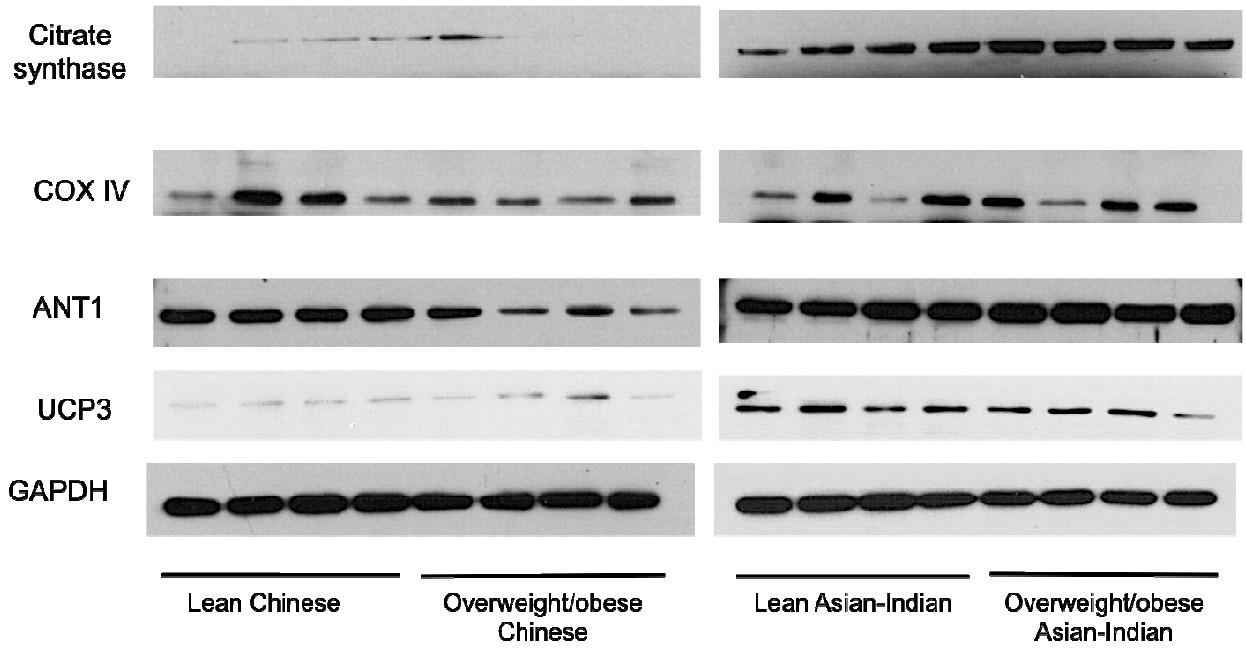


Figure 6.6 Western blots of citrate synthase (CS), cytochrome c oxidase (COX IV), adenine nucleotide translocase 1 (ANT1), uncoupling protein 3 (UCP3) and GAPDH proteins in Chinese and Asian Indian myotubes of, stratified into lean and overweight/obese groups.

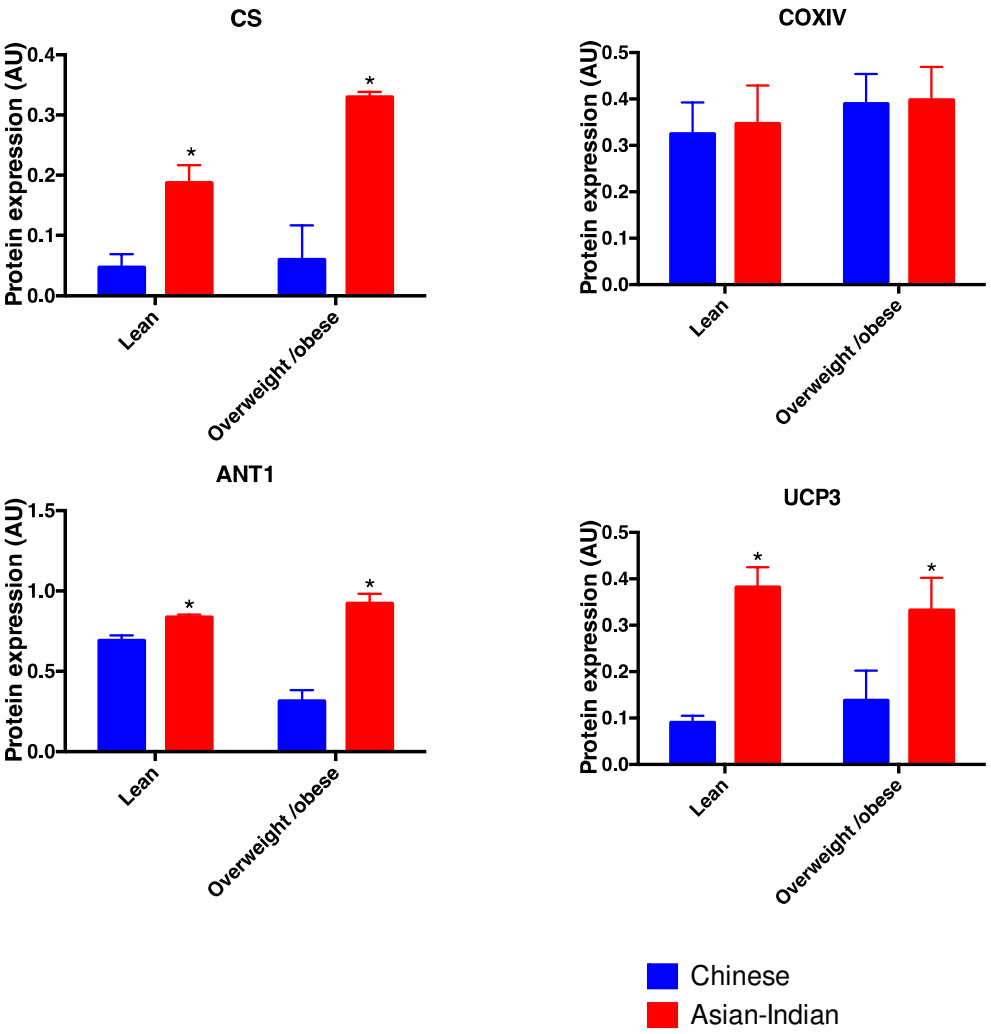


Figure 6.7 Densitometric analysis of citrate synthase (CS), cytochrome c oxidase (COXIV), adenine nucleotide translocase (ANT1) and uncoupling protein (UCP3) protein levels in primary human myotubes of Chinese and Asian Indians, stratified into lean and overweight/obese groups. Graph displays mean ± SD in arbitrary units (AU), normalized against GAPDH protein levels. *P < 0.05.

6.3.4 Mitochondrial respiration in Chinese and Asian-Indian myotubes

Proton leak and ATP turnover was not significantly associated with obesity or ethnicity (Figure 6.8). Upon stratification into lean and overweight/obese groups, we observed a trend of greater maximal respiration (178.27 ± 86.82 vs. 110.08 ± 28.72, P=0.086) and spare respiratory capacity (123.41±83.87 vs. 63.95 ± 28.24, P=0.086) in lean Asian-Indians than Chinese, while no differences were observed in the overweight/obese state. Within the Asian Indians, there was a trend of higher basal respiration in lean compared to overweight/obese individuals (54.86 ± 14.79 vs. 42.70 ± 6.31, P=0.068). Maximal respiration was significantly greater in lean Asian-Indians compared to overweight/obese counterparts (178.27±86.82 vs. 109.15 ±34.15, P=0.028).

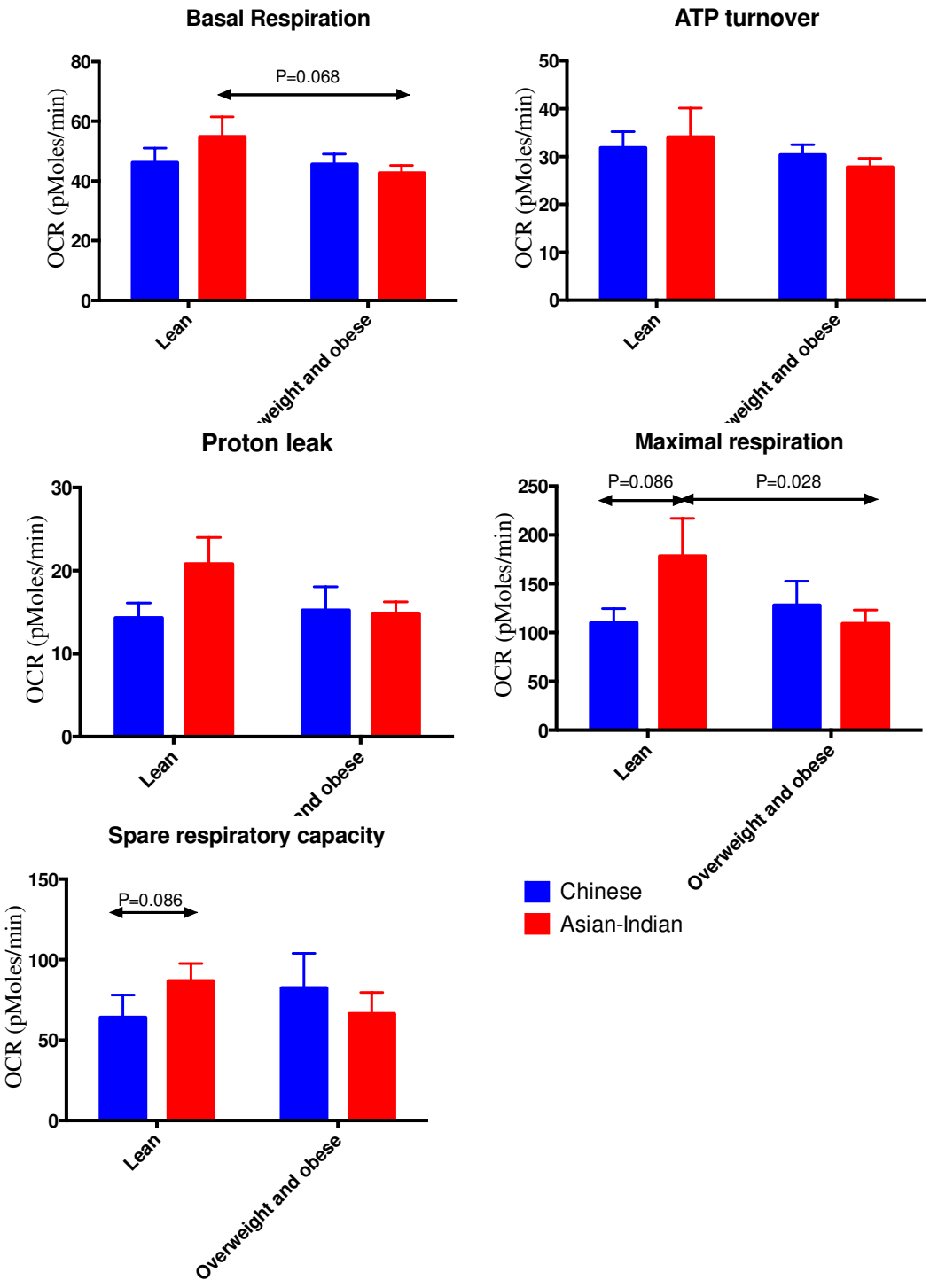


Figure 6.8 Parameters of mitochondria respiration in Chinese and Asian-Indians myotubes stratified into lean and overweight/obese groups. Data are expressed as mean ± SD.

6.3.5 Correlations between residual resting energy expenditure and skeletal muscle characteristics in Chinese and Asian-Indian myotubes

Figure 6.9 shows the relationships between residual REE (difference between REE measured and REE predicted on the basis of FFM and fat mass) with mitochondrial respiration parameters. No significant associations were observed between residual REE with any of the mitochondrial function parameters in Chinese and Asian-Indians (Figure 4). Given the putative role of uncoupling proteins UCP3 and adenine nucleotide translocase (ANT1) in thermogenesis, it was interesting to examine their protein levels in relationship to energy expenditure. Residual REE did not correlate significantly with UCP3 protein levels and ANT1 protein levels in Chinese and Asian-Indians (Figure 6.10).

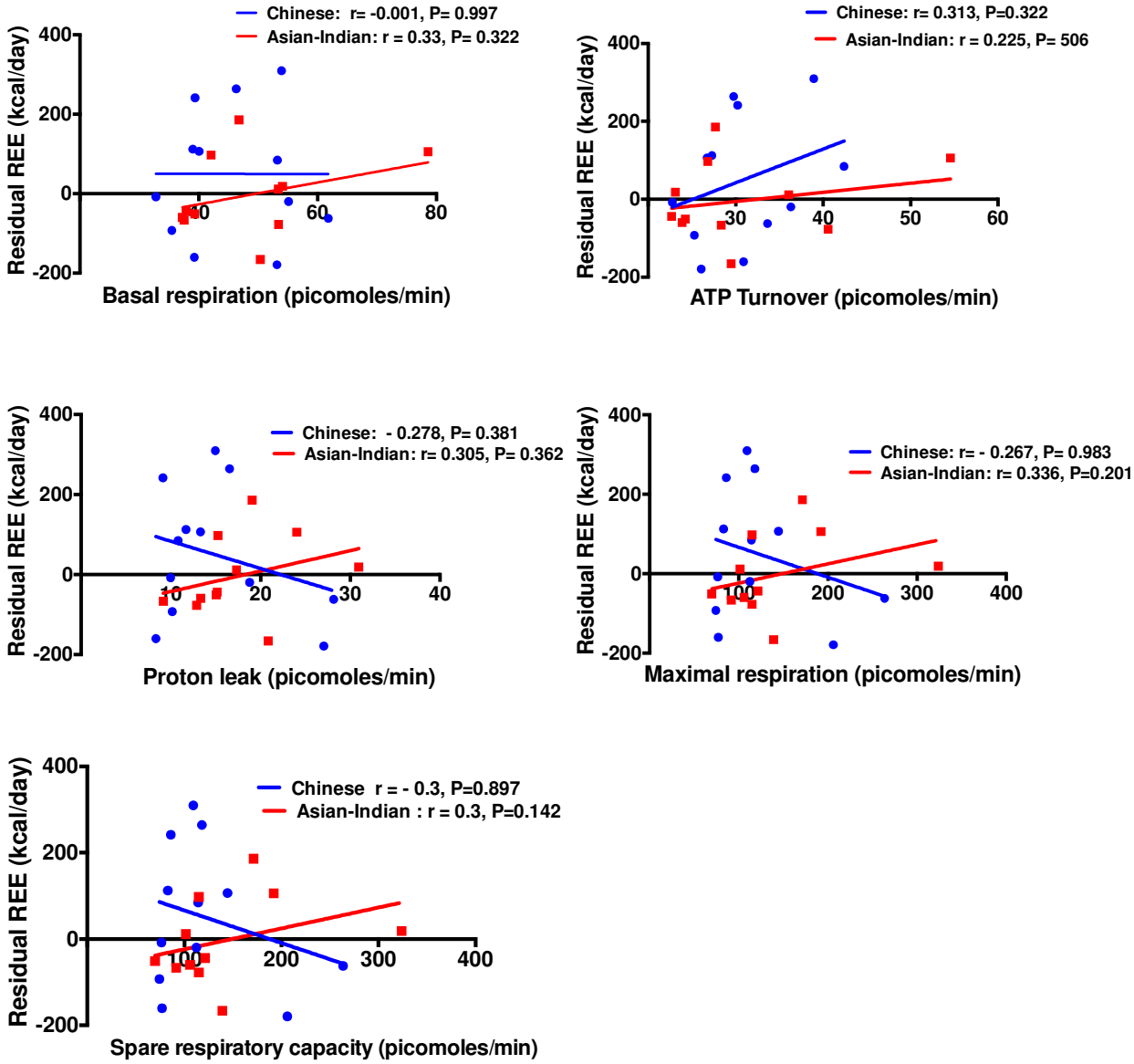


Figure 6.9 Relationships between residual resting energy expenditure (difference between resting energy expenditure measured and resting energy expenditure predicted on the basis of fat-free mass and fat mass) and mitochondrial respiration parameters in Chinese and Asian Indian myotubes.

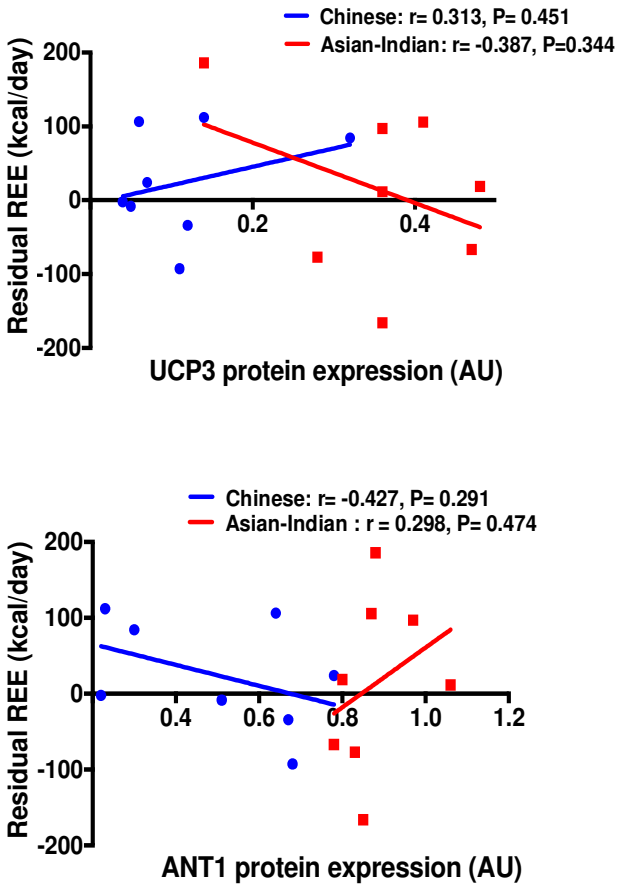


Figure 6.10 Relationships between residual resting energy expenditure (difference between resting energy expenditure measured and resting energy expenditure predicted on the basis of fat-free mass and fat mass) with uncoupling protein (UCP3) and adenine nucleotide translocase (ANT1) protein levels in Chinese and Asian Indian myotubes

6.3.6 Correlations between metabolic flexibility and mitochondrial respiration in Chinese and Asian-Indian myotubes

In Figure 6.11, RQ (iAUC) positively correlated with proton leak in Asian-Indians (r=0.648, P=0.043) but not Chinese (r=0.049, P=0.88), maximal respiration in Asian-Indians (r=0.794, P=0.006) but not Chinese (r= -0.231, P=0.471) and spare respiratory capacity in Asian-Indians (r=0.697, P=0.025) but not Chinese (r=0.084, P=0.795).

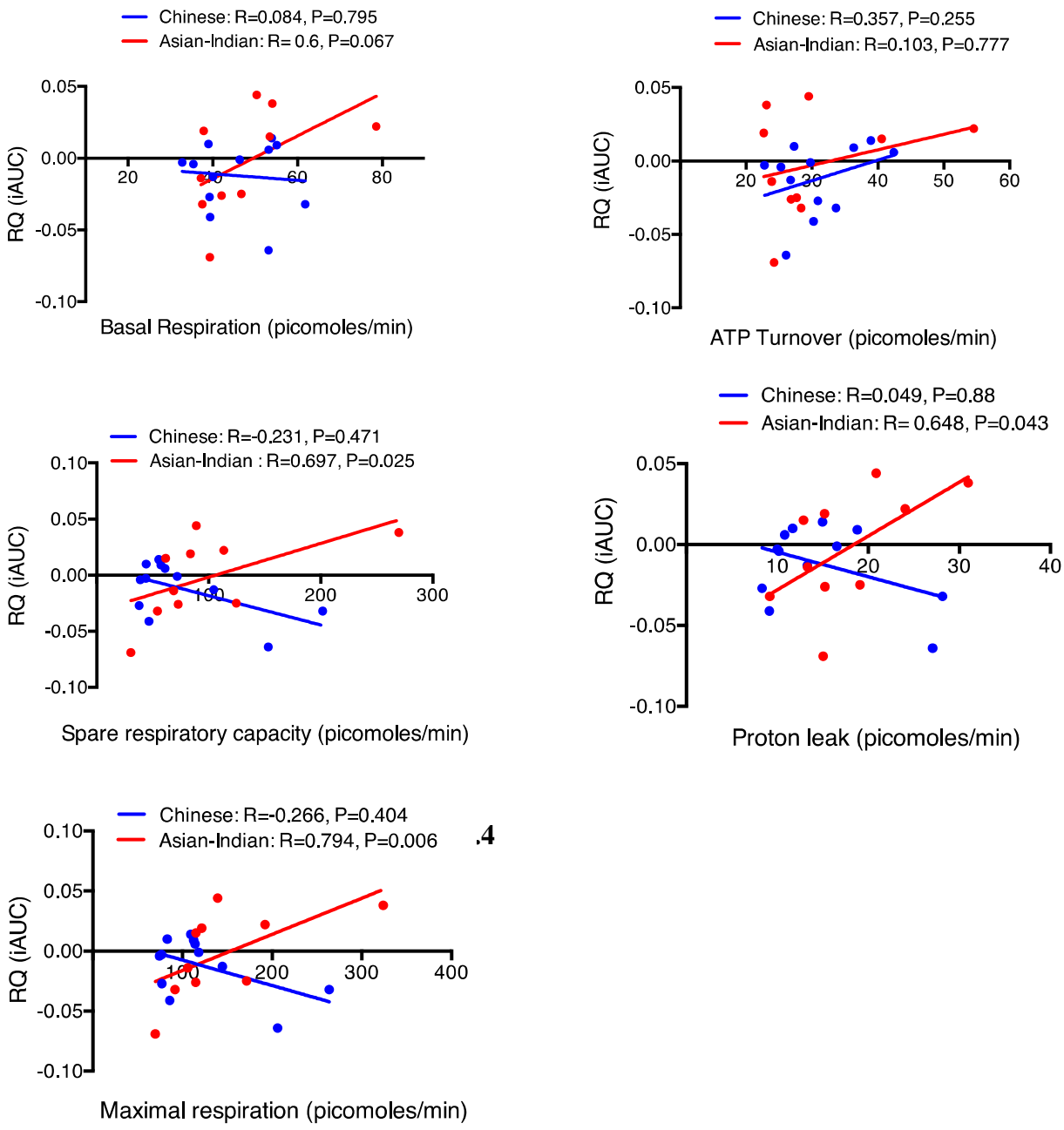


Figure 6.11 Relationships between RQ (iAUC) and mitochondrial respiration parameters in Chinese and Asian Indian myotubes.

6.4 Discussion

We demonstrate that primary human myotube is a better in vitro experimental model than myoblast for studying energy metabolism and the regulation of UCPs. We report four main findings from this study: 1) REE adjusted for fat-free mass and fat mass did not significantly correlate with mitochondrial function; 2) RQ (iAUC) positively correlated with basal and maximal respiration in Asian-Indians but not in Chinese, 3) Asian-Indians had greater skeletal muscle expression of oxidative and uncoupling proteins (UCP3, ANT1 and citrate synthase) and greater mRNA level of PGC1A, the key transcriptional regulator of mitochondrial biogenesis and oxidative phosphorylation than Chinese, irrespective of their obesity status; 2) Asian-Indians had greater mitochondrial maximal respiration and spare respiratory capacity than Chinese only in lean but not overweight/obese individuals. Collectively these data indicate that greater mitochondrial oxidative capacity in lean Asian-Indians compared to Chinese may explain the greater MF observed in the former.

6.4.1 Primary human myotube: an in-vitro model for studying energy metabolism

We demonstrate that primary human myotube is a better in vitro experimental model than myoblast for studying energy metabolism and the regulation of UCPs due to upregulation of UCP2 and UCP3 in myotubes compared to myotubes. Our results are consistent with earlier studies that showed that C2C12 mouse and L6 rat myoblast cell lines expressed extremely low levels of UCP3. Detectable levels of UCP3 were present only when the cells were

differentiated and acquired a myotube phenotype as the UCP3 promoter activity is dependent on MyoD, the myogenic determining factor(244). Human primary myotubes is a widely used in vitro model system to study energy metabolism under standardized conditions and metabolic disorders as human primary muscle cells express traits known from in vivo muscles. Another advantage is that the extracellular environment can be controlled precisely and kept constant over time, which allows the studying of genetic and epigenetic influences without systemic homeostatic regulatory components from the nervous and endocrine environment(142, 245, 246). Insulin resistance in skeletal muscle in vivo is associated with reduced lipid oxidation and lipid accumulation. According to Gaster et al, myotubes established from type 2 diabetic subjects expressed reduced palmitic acid oxidation, basal glucose uptake and insulin-stimulated glycogen synthase activity compared with control myotubes, which suggest that the diabetic phenotype might be intrinsic to the muscle cells themselves and is conserved in vitro by either genetic, epigenetic factors(247, 248). Boyle and colleagues demonstrated that myotubes from lean subjects has increased mitochondrial respiration (state 3) in the presence of palmitoyl carnitine compared to obese subjects, indicating that skeletal muscle of obese individuals inherently lacks metabolic inflexibility in response to lipid exposure(145). Therefore, this in vitro model provides an experimental system enabling us to study potential intrinsic mechanisms to the skeletal muscle that may explain variability in energy expenditure and metabolic flexibility.

6.4.2 Relationship between skeletal muscle metabolism and resting energy expenditure

This study shows that the remaining variability in REE after adjustment for FFM and fat mass cannot be explained by variation in mitochondrial function, in particular proton leak. Our data does not support the study of Rolfe and Brand who reported that proton leak in the skeletal muscle is a significant contributor, accounting for 17-21% of REE in rodents(243). In humans, studies have reported the link between proton leak and adaptive thermogenesis. Wijers and colleagues showed that in lean male subjects, 50% of the variability in nonshivering thermogenesis upon mild cold exposure (16°C) could be explained by mitochondrial proton leak in permeabilized muscle fibers. In a study by Lebon and colleagues, infusion of triiodothyronine (T3), a known factor involved in thermogenesis, in human subjects increased TCA flux in combination with unchanged ATP synthesis, indicative of increased mitochondrial proton leak (249). These data suggests that in humans, skeletal muscle may be more important in regulating adaptive thermogenesis rather than resting energy metabolism. Indeed, the muscle tissue can only account for 20-30% of total resting oxygen uptake, however it can account for up to 40-50% increase in whole body oxygen uptake after epinephrine or ephedrine infusion(72). High metabolic rate organs (brain, kidney, heart and liver) contribute a disproportionately larger fraction of REE than their fractional volume of the body compared to the muscle. Thus the pathways contributing to the metabolic rate of these organs may be of more relevance to the study of REE. In addition, other ATP consuming processes such as protein turnover, ion cycling across plasma membranes (Ca^{2+} and

Na^+/K^+ ATPase) and substrate cycling that were not examined in this study, could contribute to variation in REE. Another possibility is that myotube cultures may not be a suitable in vitro experimental model for the study of energy metabolism. The drawback of cell cultures in general is that cells change their phenotype, which may alter the expression of certain genes and compromise the phenotypic expression of the disease. Indeed, the comparison of transcriptome between human myotubes and skeletal muscle tissue revealed a significant downregulation of genes involved in key metabolic pathways such as energy metabolism (UCP3), oxidative phosphorylation (complex I, II, III and IV), fatty acid oxidation (CPT1B) and glycolysis(250).

We also showed that protein level of UCP3 was not correlated with REE, inconsistent with the concept of a thermogenic role for uncoupling proteins. Schrauwen et al reported that in Pima Indians, UCP3 mRNA positively correlated with REE, indicating that UCP3 may be a determinant of energy expenditure(119). However, the question of whether the relationship was also observed at the protein abundance or activity level was not addressed. Our finding is consistent with many other studies reporting the lack of association between UCPs and REE. Bao and colleagues reported that UCP2 and UCP3 mRNA levels were not correlated with REE in adults(127). Yanovski et al also found no significant association between UCP2 genotype and REE in children(251). After 10 weeks of weight loss on low caloric diet in T2D subjects, diet induced changes in UCP3 protein was not significantly correlated with REE, but was associated with changes in skeletal muscle fatty-acid binding protein content, suggesting a role of UCPs in fatty acid

metabolism rather than regulation of energy expenditure(252). Significant increases in UCP2 and UCP3 gene expression are observed during fasting and severe food restriction, conditions in which metabolic rate decreases(253). Boss et al. (26) have detected a significant positive correlation between UCP3 mRNA and circulating free fatty acids in human muscle from obese patients. We also examined the association between ANT1 protein and REE but observed no significant associations. Brand and colleagues reported that the adenine nucleotide carrier catalyzed half to two-thirds of the basal proton conductance of Drosophila mitochondria and may be an important contributor to metabolic rate(103). However, ANT1 may have a greater role in catalyzing ADP–ATP exchange rather than proton leak. In humans, mutations in the heart and muscle isoform of ANT1 were found to be associated with autosomal-dominant progressive external opthalmoplegia (adPEO) clinically characterized by increased serum lactate, exercise intolerance, ptosis and muscle weakness. Muscle biopsies show over proliferation of mitochondria and mild reduction of oxidative phosphorylation enzymes(254). Mielke et al recently reported that acetylation of ANT1 in vivo in human skeletal muscle was associated with decreased ADP affinity, altered OXPHOS and insulin sensitivity(255).

Furthermore, we observed higher UCP3 protein levels in Asian-Indians than Chinese, which is counterintuitive since Asian-Indians have lower REE. To our knowledge, only one study has examined the contribution of UCP3 to ethnic differences in REE. Kimm et al associated a UCP3 exon 5 polymorphism with significantly lower REE in African American women with

the CC genotype than in those with the TT genotype, while this effect was absent in White women, thereby suggesting that this variant is responsible for the lower REE observed in African Americans. However, nothing is known about the effect of the exon 5 variant on UCP3 protein levels or function. The implications of higher UCP3 in Asian-Indians are not exactly known, however we postulate that upregulation of UCP3 may be a compensatory mechanism to reduce reactive oxygen species (ROS) and protect cells from oxidative damage to prevent the greater development of insulin resistance. We have shown that within Asia, Asian-Indians exhibit the greatest insulin resistance and Chinese the least. Our hypothesis is supported by the study of Aguer and colleagues who demonstrated that myotubes from obese non-diabetic subjects with a family history of T2D have increased proton leak, lower ROS and similar oxidative stress level compared to obese non-diabetic subjects without a family history of T2D.

6.4.3 Relationship between skeletal muscle metabolism and metabolic flexibility

MF was positively associated with mitochondrial basal respiration and FCCP induced maximal respiration which is consistent with previous studies. Maximal respiration reflects the maximal mitochondrial oxidative capacity of the electron transport chain and the upstream substrate translocases and dehydrogenases and is referred to as maximal uncontrolled mitochondrial oxidative capacity(168). Phielix et al reported that the rate of PCr resynthesis (ie halftime of recovery), which reflects in vivo mitochondrial oxidative capacity or function was associated with insulin stimulated glucose oxidation

and MF. Subjects with a family history of T2D had an impaired ability to increase fatty acid oxidation in response to high fat meal and this was associated with impaired activation of genes involved in oxidative and lipid metabolism such as peroxisome proliferator-activated receptor coactivator-1 α (PGC1A) and fatty acid translocase (FAT/CD36)(137) Metabolic switching represented by dynamic changes in fat oxidation in primary human myotubes mirror the MF phenotype of the cells' donor, supporting the hypothesis that MF is an intrinsic characteristic of skeletal muscle. In contrast, Wijers et al found that in vivo mitochondrial capacity was related to basal RQ but not MF, suggesting that reduced mitochondrial function does not negatively impact the ability of skeletal muscle to switch substrates, rather it is primarily responsible for basal substrate utilization. Gaster and colleagues reported that suppression of glucose oxidation in response to palmitate in the presence of insulin was similar in myotubes established from lean, obese, and type 2 diabetic donors, indicating that metabolic inflexibility is not an intrinsic defect in skeletal muscle, but based the inability to vary extracellular fatty acid concentrations during insulin stimulation(146).

Lean Asian-Indians had higher skeletal muscle mitochondrial OXPHOS capacity as demonstrated by higher mitochondrial maximal respiration, higher protein abundance of citrate synthase and mRNA of PGC1A compared to their Chinese counterparts. Our results are consistent with those of Nair and colleagues who reported that Asian-Indians had increased skeletal muscle capacity for OXPHOS and mitochondrial DNA copy number than Northern European Americans(256). Hall et al also showed that South-Asians exhibited

greater oxidative and lipid metabolism capacity compared to Europeans(257). The potential implication of a higher oxidative capacity on energy metabolism in lean Asian-Indians is not clear at this time, however we postulate that it promotes a metabolic shift towards increased glucose oxidation in muscle, contributing to greater MF observed in Asian-Indians.

In summary, these data indicate that lower REE in Asian-Indians than Chinese is not the consequence of higher skeletal muscle expression of UCP3 in Asian-Indians. Skeletal muscle may not have the intrinsic capacity for regulating resting energy metabolism via mitochondrial proton leak. Greater maximal respiration in lean Asian-Indians than Chinese could explain the greater MF observed in Asian-Indians.

Chapter 7 . Characterization of fat mass and obesity associated (FTO) gene in skeletal muscle energy and nutrient metabolism and its epigenetic link to obesity

7.1 Introduction

Several single nucleotide polymorphisms (SNPs) that cluster in the first intron of fat mass and obesity associated (FTO) gene are associated with obesity in genome-wide association studies (GWAS). Human studies to date suggest that the association between SNPs in FTO and BMI is predominantly driven by increased energy intake, but not energy expenditure(258-260). Individuals homozygous for the obesity-risk allele exhibit greater ad libitum food intake(261), particularly dietary fat consumption(262), increased appetite and reduced satiety(263), poor food choices(264) and eating habits and loss of control over eating(265) than those homozygous for non-risk allele. Evidence from rodent studies suggests that FTO is nutritionally regulated and can influence food intake, which is consistent with human studies. FTO mRNA and protein levels are downregulated by essential amino acid deprivation in mouse cell lines(266). Within the arcuate nucleus of the hypothalamus, 48-h fasting reduced FTO expression, whereas prolonged exposure to high fat diet increased FTO expression(267). In addition, selective alteration of FTO levels in the arcuate nucleus influenced food intake(171). FTO is ubiquitously expressed across multiple tissues, but is most highly expressed in brain, especially the hypothalamus, a region with a key role in the control of food intake(267).

Recently, several animal studies have shown the effect of FTO on body weight regulation and energy expenditure, suggesting the peripheral role of FTO in energy homeostasis(158, 159). FTO null mice exhibit significant reduction in adipose tissue and a decreased propensity to gain weight on a high-fat diet (HFD), compared with their wild-type littermates. The lean phenotype developed as a consequence of increased energy expenditure and systemic sympathetic activation, despite decreased physical activity and hyperphagia(158). Mice with a missense loss of function mutation (I367F) in FTO also displayed a lean phenotype with an increased metabolic rate but no change in physical activity or food intake(159). However to date, the molecular mechanisms by which FTO regulates energy metabolism in human skeletal muscle remains to be elucidated.

The disadvantage of using genetic approaches, such as gene deletion/ gene silencing and lost-of-function mutations is that they may not accurately reflect the actual physiological conditions(171). Knockout of the FTO gene in animal model frequently results in severely compromised organism, which is associated with increased postnatal death and growth retardation(158). This, however, is not observed in human population carrying the FTO risk alleles. Human SNPs involve an impairment of function, rather than a complete loss of function. In addition, genetic manipulation may also result in altered expression of other genes in the same genomic region through long range interactions, such as IRX3 and, potentially, other genes.

To overcome these limitations, we propose using a chemical probe approach, where small molecule that selectively inhibits FTO demethylase activity will be used to dissect the molecular mechanisms of FTO. The chemical probe approach is complementary to, and has advantages over genetic methods, as it would allow a temporary, dose-dependent reduction of FTO catalytic activity, without affecting other potential biological functions of FTO.

FTO is a Fe (II) and 2-oxoglutarate (2OG) dependent demethylase, homologous with the E.coli DNA repair protein AlkB and the mammalian homologues ALKBH2 and ALKBH3(267). In vitro, recombinant FTO catalyzes the oxidative demethylation of 3-methylthymine in single-stranded DNA, 3-methyluracil in RNA(267, 268) and N6-methyladenine (m6A)(269) in RNA, suggesting a physiological role for FTO in nucleic acid repair or modification. Of special interest is the m^6A modification, which is one of the most common and abundant post-transcriptional modification in mRNA. It has been identified in more than 7000 genes in human, where it is enriched near stop codons and in the 3'-UTR of mRNA, hence suggesting a possible involvement in the regulation of gene expression(270). This modification has also been suggested to affect RNA processing, RNA transport and translation efficiency(271). However, it is unclear if and how the m6A demethylation activity of FTO is linked to human obesity.

The aim of this study is to characterize the function of FTO in skeletal muscle metabolism and examine how the N6-methyladenosine (m^6A) demethylation activity of FTO contributes to its metabolic effects and obesity using two

approaches a) genetic approach by gene silencing and b) chemical probe approach using a novel pharmacologic inhibitor (CA) of FTO catalytic activity. We will be focusing on 4 metabolic pathways : 1) Energy metabolism, 2) fatty acid metabolism (β-oxidation and de novo lipogenesis), 3) TCA cycle and oxidative phosphorylation and 4) Akt insulin signaling pathway.

7.2 Material and methods

7.2.1 Subjects

Primary human myoblast were initiated from satellite cells of vastus lateralis obtained from 4 healthy lean Chinese men with normal insulin sensitivity, FPG <7.0 mmol/L and no prior history of T2DM. Subject details are described in Section 2.2

7.2.2 Discovery of potent and subfamily-selective small molecule probe of FTO

Prior to this study, there was no report of small molecule that is able to selectively inhibit the demethylase activity of FTO. Prof Esther Woon's lab embarked on a probe discovery programme, where they employed an innovative drug discovery strategy called Dynamic Combinatorial Mass-Spectrometry (DCMS)(272). This technique uses a self-assembly process to spontaneously generate a dynamic library of chemical compounds. Compounds that fit best into the catalytic site of FTO will be preferentially formed and be detected by Protein Mass-Spectrometry. This method led to the first discovery of several promising FTO inhibitors, which has been successfully patented. One inhibitor in particular, CA, satisfies all the important requirements for use as a FTO functional probe : 1) CA is able to inhibit the m6A demethylase activity of FTO (IC50 = 0.8μM), 2) CA is highly selective for FTO against other structurally similar proteins and exhibits 30-fold to 130-fold selectivity for FTO over structurally related subfamily members such as AlkB, ALKBH2, ALKBH3 and ALKBH5; 3) CA is active

in cells, with low cytotoxicity. MTT assay with HeLa cells showed that >80% of the cells remain viable after treatment with 100□M of CA and HeLa cells that were treated with CA showed a significant increase in the level of m6A modification in the total mRNA compared to the untreated control.

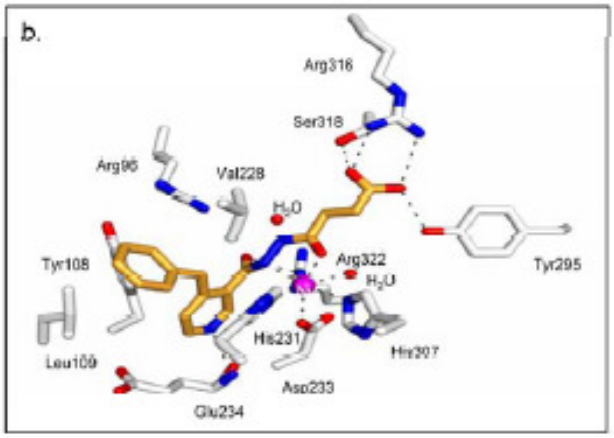


Figure 7.1 A crystal structure of FTO in complex with CA

7.2.3 MTT assay

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma). Primary human myoblasts were seeded at a concentration of 5 X 10^4 cells/ml onto 96-well plates and were allowed to adhere overnight. After 24h, cells were treated with CA in the concentration range of 10 -100 µM for 72 h with untreated cells acting as controls. After 72 h, medium was replaced with 100 µl of 0.5 mg/ml MTT

each well and was incubated for another 4 h. Formazan crystals formed were solubilized in 10% sodium dodecyl sulfate with 0.01 M hydrochloride acid (100 µl) for 4 h and the absorbance was measured at 570 nM with a reference wavelength of 650 nM using a micro-plate reader (Multiskan Spectrum, ThermoElectro Corporation, Waltham, MA, USA). Cell viability (%) was calculated using the formula: [(Mean absorbance of the sample – reference absorbance)/ (Mean absorbance of the control)]

7.2.4 Small interfering RNA (siRNA) transfection of FTO and CA treatment in primary human myotubes

Gene silencing by siRNA was performed using siTRAN 1.0 (Origene, Sweden) onto cells cultured in 6-well plates. Differentiation media was changed to PS-free growth medium on day 2 of myotube differentiation. On day 3, siRNAs against FTO (5 nM) (Origene, Sweden) were transfected using siTRAN in serum-free DMEM (incubating time >24 hr). Myotubes were then washed with PBS and maintained in differentiation medium. To study the effects of acute and chronic exposure to CA, myotubes were treated with 50 µM and 100 µM CA for 6 h and 24 h respectively on day 6 of differentiation. For insulin treatment experiments, cells were deprived of serum for 4 hours before stimulation with insulin (100 nM) for 30 min before harvest. Cells were used for experiments 6-7 days following differentiation.

7.2.5 Quantification of mRNAs

Total RNA samples from cell cultures were extracted as previously described in Section 2. mRNA levels were measured by reverse transcription followed by real time PCR in Section 2.13.

7.2.6 Western Blot

Quantification of protein abundance were performed using techniques described in Sections 2.14.

7.2.7 High resolution respirometry in CA treated myotubes

Mitochondrial bioenergetics was measured in primary human myotubes using a Seahorse XF24 Analyzer (Seahorse Biosciences, North Billerica, MA) as previously described in Section 2.12. Myoblasts were seeded on XF24 tissue culture plates at 20 000 cells/well in growth medium using 6 technical replicates of each clinical sample and induced to differentiate into myotubes. At day 5 of differentiation, 50 μM CA was added for 24 h incubation. The next day, myotubes were incubated for 45 min at 37 °C at ambient CO_2 in XF Assay Medium (Seahorse Biosciences) (pH 7.4) containing 4 mM glutamine, 1 mM pyruvate and 25 mM glucose.

7.2.8 2-deoxy-D-[3H]glucose uptake assay in CA treated myotubes with palmitate treatment

Primary human myotubes were pre-incubated in serum-free DMEM for 6 h. Cells were then incubated in PBS + 0.2 % BSA for 30 min at followed by addition of 5 μM [3H]2-deoxyglucose (0.50 μCi per well), for 10 min. After incubation with [3H]2-deoxyglucose, medium was rapidly aspirated and cells washed three times with ice-cold PBS and lysed in 200 μl of RIPA buffer, of which 100 μl was transferred to scintillation liquid and the remaining was used for protein concentration using Bradford Assay. Radioactivity was determined by liquid scintillation counting.

7.2.9 Statistical Analyses

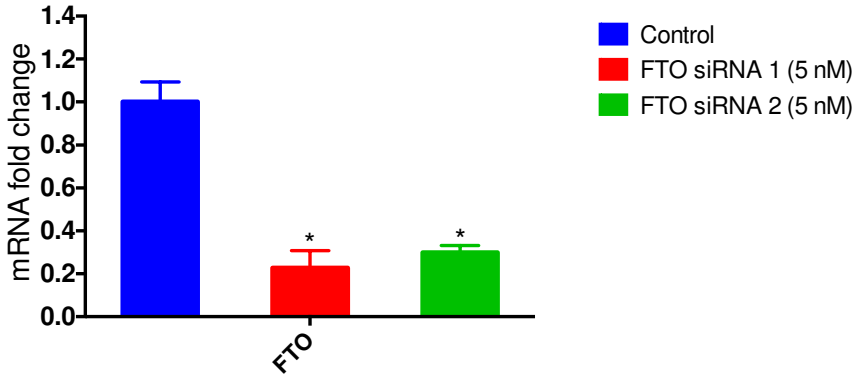
Statistical analyses were performed using IBM SPSS Statistics for Mac Version 20.0; Armonk, NY: IBM Corp(196). All statistical tests were two-sided, and a P value<0.05 was considered statistically significant. Comparisons of mitochondrial respiration parameters, mRNA and protein expression levels between FTO knockdown myotubes and controls and between CA treated myotubes and controls were conducted using Kruskal-Wallis test.

7.3 Results

7.3.1 FTO knockdown in human myotubes

In FTO-deficient myotubes, FTO mRNA levels were reduced to <30% and FTO protein levels were reduced to <40% (Figure 7.2). Cells were viable and showed no obvious change in their phenotype.

A



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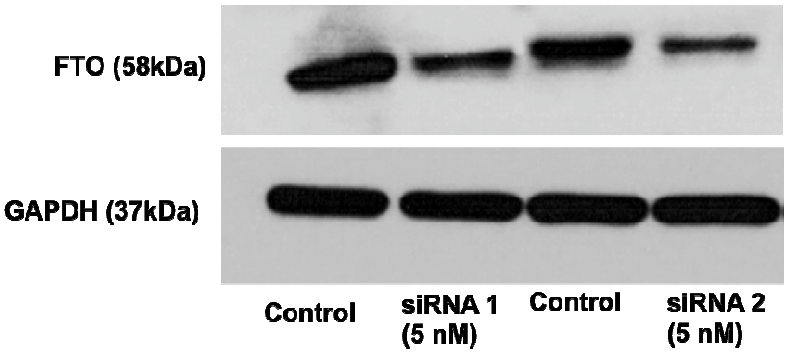


Figure 7.2 Knockdown of FTO in primary human myotubes (A) RT-PCR showed decreased FTO mRNA levels in both FTO-specific siRNA transfections. Data are expressed as mean ±SD. *, P<0.05 (B) Reduced FTO protein levels were revealed by western blot in both FTO-specific siRNA transfections.

7.3.2 MTT Cytotoxic Assay

MTT assay with primary human myotubes showed that >90% of the cells remain viable even after treatment with high dose of CA (100 μM), showing that CA is low is cytotoxicity (Figure 7.3).

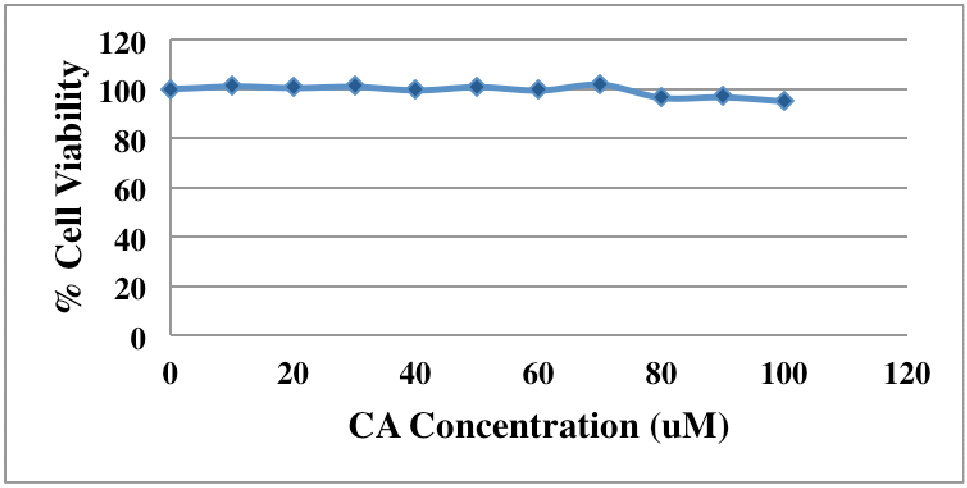


Figure 7.3 Effects of CA on the viability of primary human myotubes, as determined by MTT cytotoxicity assay.

7.3.3 Gene expression in FTO knockdown primary human myotubes

The mRNA level of uncoupling protein 3 (UCP3), involved in proton leak (Figure 7.4A) was significantly induced by 2.2 fold in FTO deficient myotubes compared with control myotubes. Carnitine palmitoyltransferase 1B (CPT1B) essential for β-oxidation of fatty acid was significantly upregulated while acetyl CoA carboxylase (ACC1) and fatty acid synthase (FASN) that promotes the synthesis of fatty acid were significantly downregulated (Figure 7.4B). The expression of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC1A), the driver of mitochondrial biogenesis and oxidative phosphorylation (OXPHOS), was also significantly increased in FTO-deficient cells by approximately 2-fold (Figure 7.4C). There was no significant change in the expression of isocitrate dehydrogenase (IDH3G), which is involved in tricarboxylic acid cycle (TCA), although the expression of citrate synthase (CS) was significantly downregulated (Figure 7.4C).

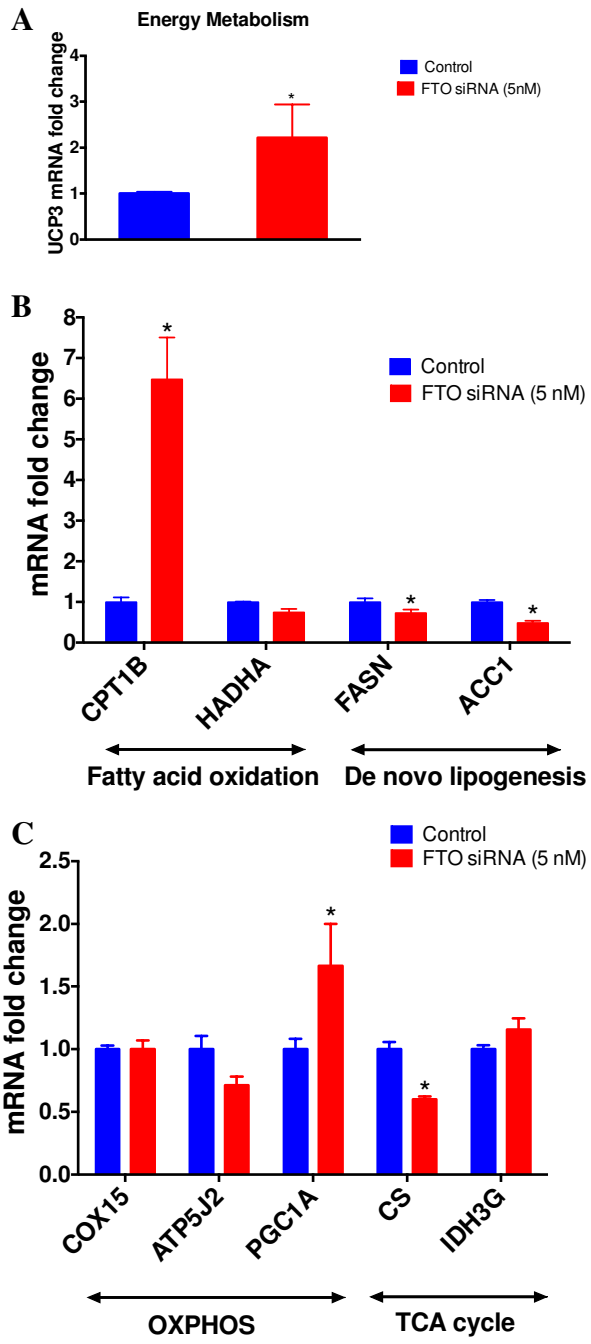


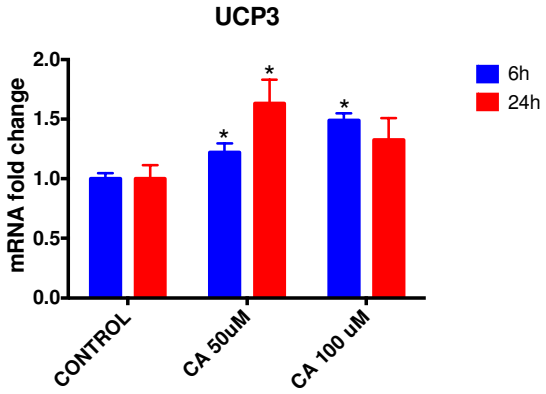
Figure 7.4 The effects of FTO deficiency on the expression of metabolic genes in primary human myotubes involved in (A) energy metabolism, (B) fatty acid oxidation and de novo lipogenesis, (C) oxidative phosphorylation (OXPHOS) and TCA cycle. Data are expressed as expressed as mean ±SD. *, P<0.05 versus control. Abbreviations used: UCP3, uncoupling protein; CPT1B, carnitine palmitoyltransferase 1B; HADHA, hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase; FASN, fatty acid synthase; ACC1, Acetyl-CoA Carboxylase 1; COX15, Cytochrome c oxidase assembly protein

COX15 homolog; ATP5J2, ATP Synthase, H+ Transporting, Mitochondrial Fo Complex, Subunit F2; PGC1A, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; CS, citrate synthase; IDH3G, isocitrate dehydrogenase 3 (NAD+) gamma

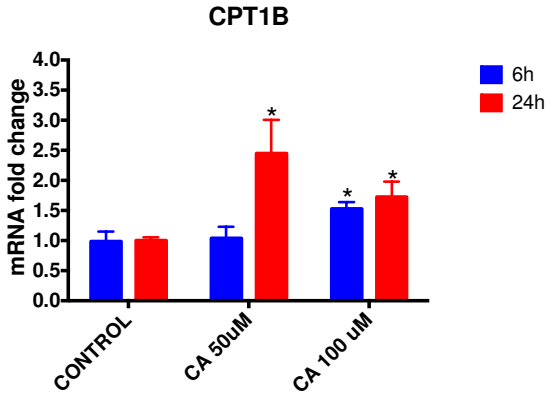
7.3.4 Gene expression in FTO inhibitor (CA) treated primary human myotubes

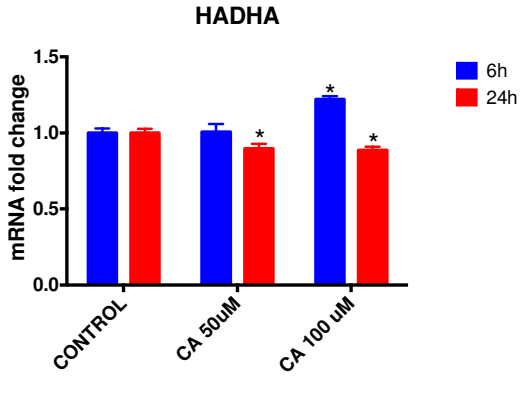
Similar to the FTO knockdown myotubes, CA treatment induced the gene expression of UCP3 (Figure 7.5A) and CPT1B (Figure 7.5B) at both 6h and 24h of exposure. mRNA level of hydroxyacyl-Coenzyme A dehydrogenase (HADHA) involved in fatty acid oxidation increased after 6h CA treatment but decreased after 24 h CA treatment (Figure 7.5B). This is coupled with significant downregulation of genes ACC1 and FASN in a dose dependent manner (Figure 7.5C). OXPHOS genes such as ATP5J2 and PGC1A (Figure 7.5D) were significantly upregulated, although a downregulation of CS was observed (Figure 7.5E)

A

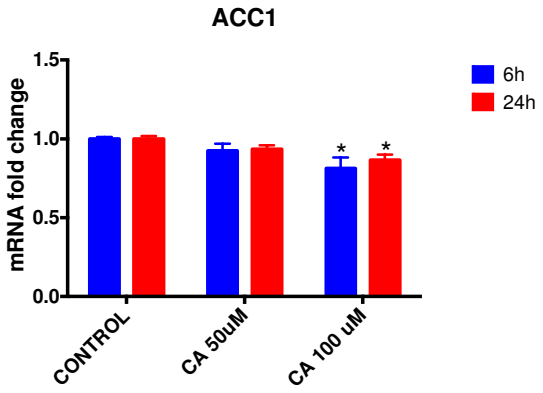

UCP3
mRNA fold change
2.0
1.5
1.0
0.5
0.0
6h
24h
CONTROL
CA 50uM
CA 100 uM

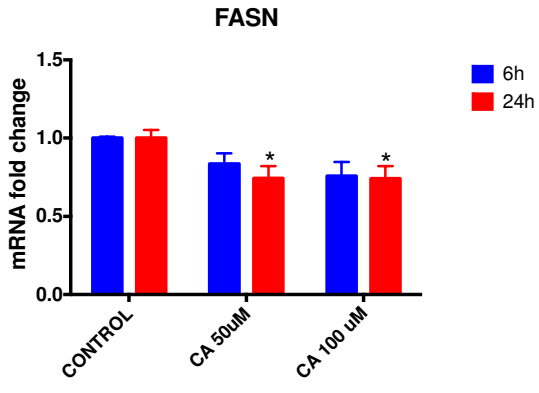
B


CPT1B
mRNA fold change
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
6h
24h
CONTROL
CA 50uM
CA 100 uM


HADHA
mRNA fold change
1.5
1.0
0.5
0.0
6h
24h
CONTROL
CA 50uM
CA 100 uM

C


ACC1
mRNA fold change
1.5
1.0
0.5
0.0
6h
24h
CONTROL
CA 50uM
CA 100 uM


FASN
mRNA fold change
1.5
1.0
0.5
0.0
6h
24h
CONTROL
CA 50uM
CA 100 uM

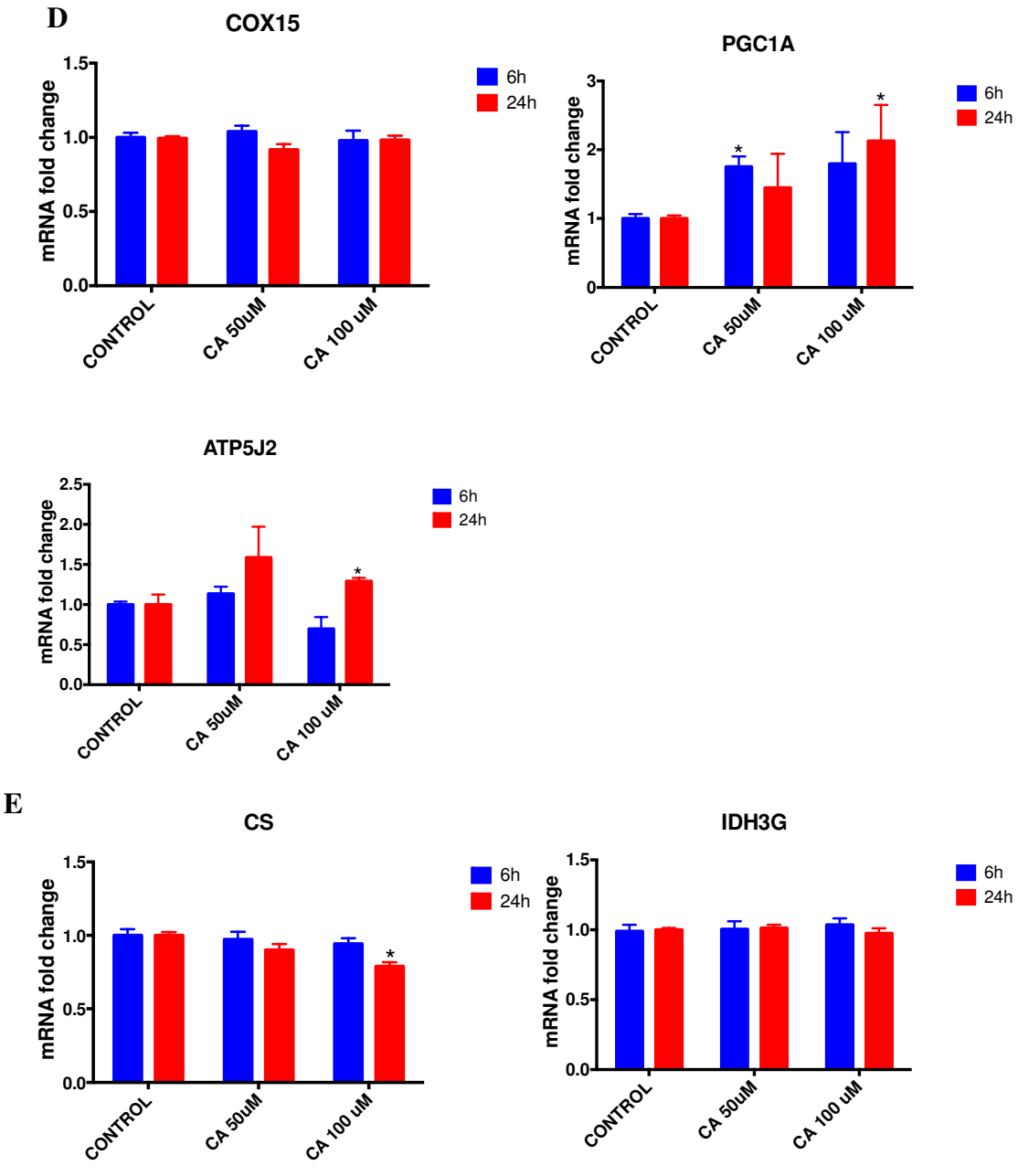
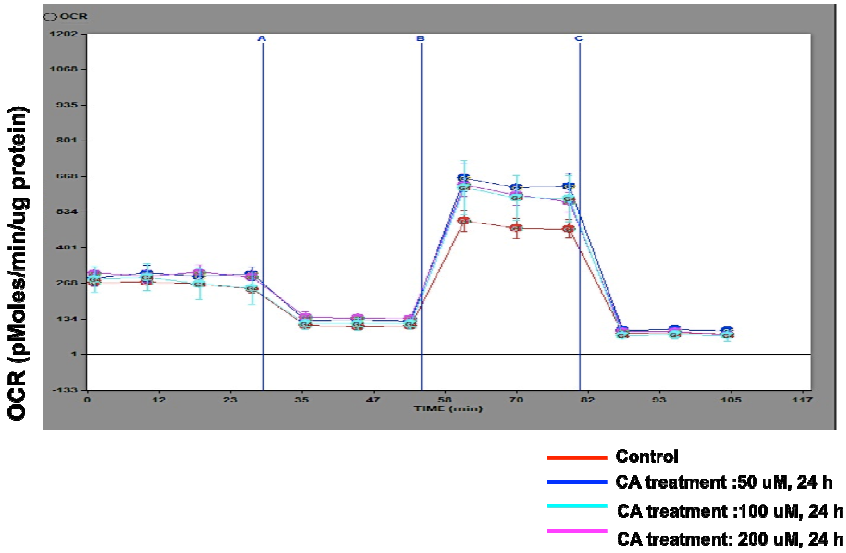


Figure 7.5 The effects of FTO inhibitor (CA) on the expression of metabolic genes in primary human myotubes involved in (A) energy metabolism, (B) fatty acid oxidation (C) de novo lipogenesis, (D) oxidative phosphorylation and (E) TCA cycle. Data are expressed as mean ±SD. *, P<0.05 versus control. Abbreviations used: UCP3, uncoupling protein; CPT1B, carnitine palmitoyltransferase 1B; HADHA, hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase; FASN, fatty acid synthase; ACC1, Acetyl-CoA Carboxylase 1; COX15, Cytochrome c oxidase assembly protein COX15 homolog; ATP5J2, ATP Synthase, H+ Transporting, Mitochondrial Fo Complex, Subunit F2; PGC1A, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; CS, citrate synthase; IDH3G, isocitrate dehydrogenase 3 (NAD+) gamma

7.3.5 Mitochondria respiration in FTO inhibitor (CA) treated primary human myotubes.

To verify whether the altered expression of mitochondrial genes was functionally relevant, we determined mitochondrial respiration rates in CA treated myotubes (Figure 7.6). Treatment of myotubes with a low dose of 50 μM CA for 24h was able to induce a maximal response in mitochondrial respiration in myotubes similarly to the higher doses (100μM and 200μM) (Figure 7.6A). A significant increase in basal respiration was observed, which is likely due to an increase in ATP turnover, rather than proton leak (Figure 7.6B). There was also a marked increase in spare respiratory capacity implying an overall improvement in mitochondria oxidative capacity. There was no significant change in non-mitochondria respiration.

A



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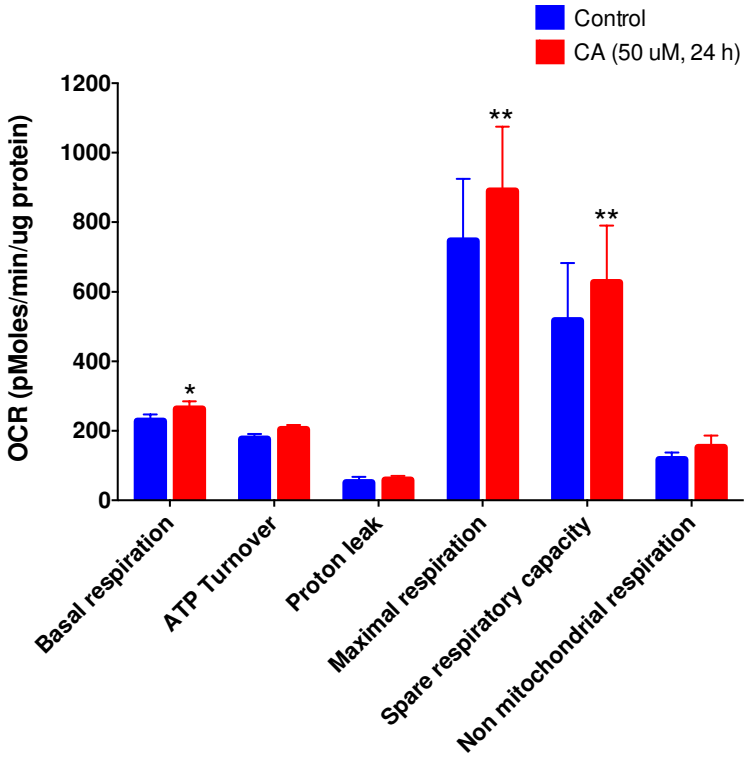


Figure 7.6 The effects of FTO inhibitor (CA) on mitochondria bioenergetic profile of primary human myotubes. (A) The oxygen consumption rate (OCR) of human myotubes when treated with 50µM, 100µM and 200µM CA for 24h. (B) FTO inhibition caused a significant increase in basal respiration and spare respiratory capacity. (*P < 0.05; **P < 0.001). Data are expressed as mean ±SD.

7.3.4 Glucose uptake and p-Akt signalling pathway in FTO inhibitor (CA) treated primary human myotubes

We further investigated the effect of CA treatment on glucose uptake and insulin sensitivity. Treated human myotubes showed an increase in basal glucose uptake rate compared to untreated control (Figure 7.7). Remarkably, CA was also able to alleviate the reduction in glucose uptake caused by palmitate acid (PA). The effect of CA does not appear to be mediated via the insulin-Akt signaling pathway, as CA treatment did not significantly modify basal and insulin-stimulated Akt (Ser473) phosphorylation (Figure 7.8). Similarly, FTO deficiency in human myotubes did not significantly modify basal and insulin stimulated Akt phosphorylation (Figure 7.9).

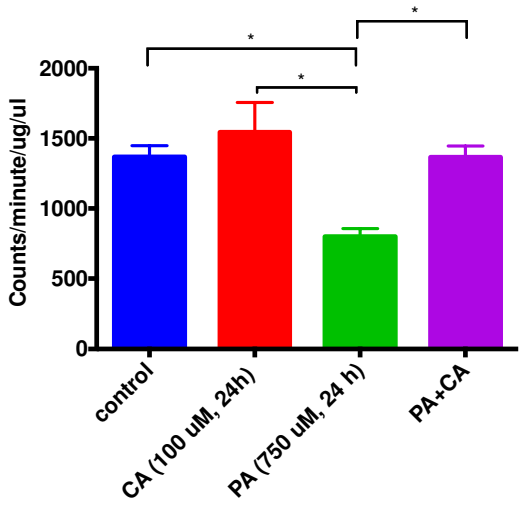


Figure 7.7 The effects of FTO inhibitor (CA) and palmitate (PA) on basal glucose uptake in primary human myotubes.

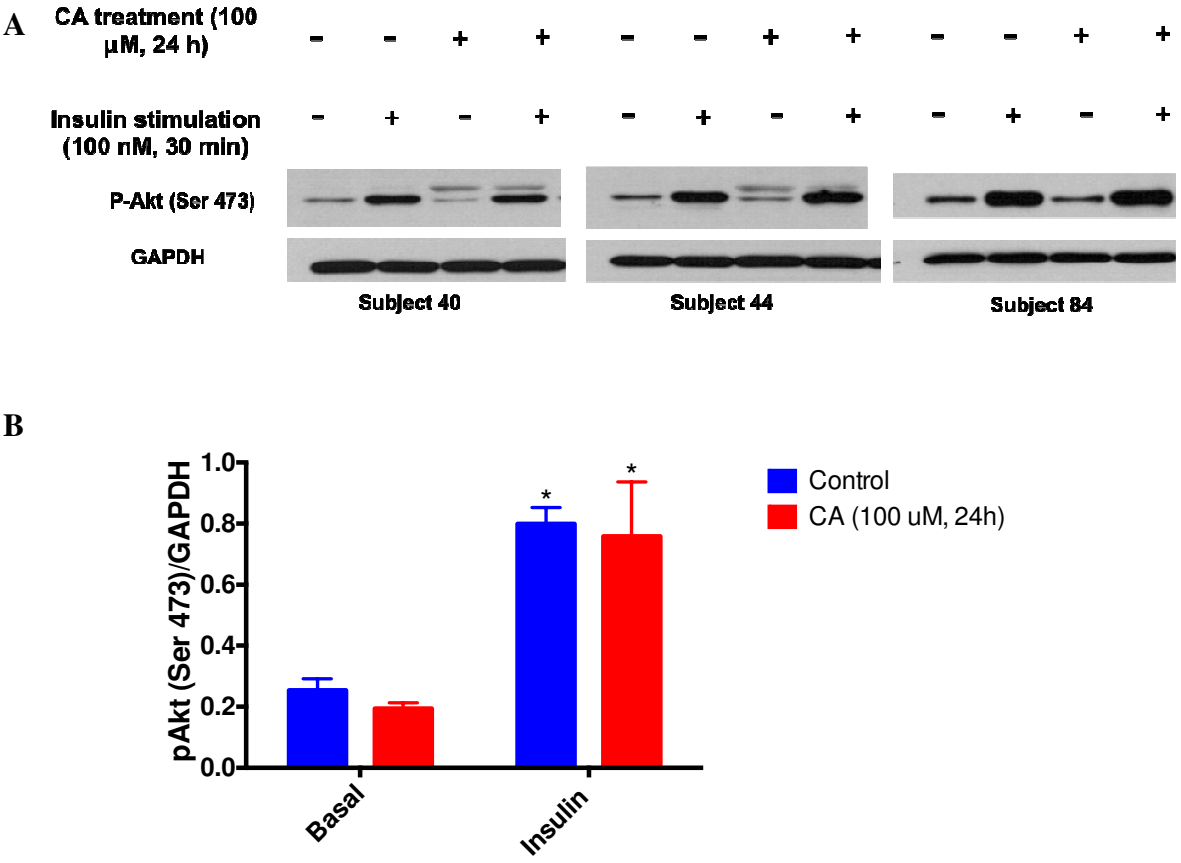
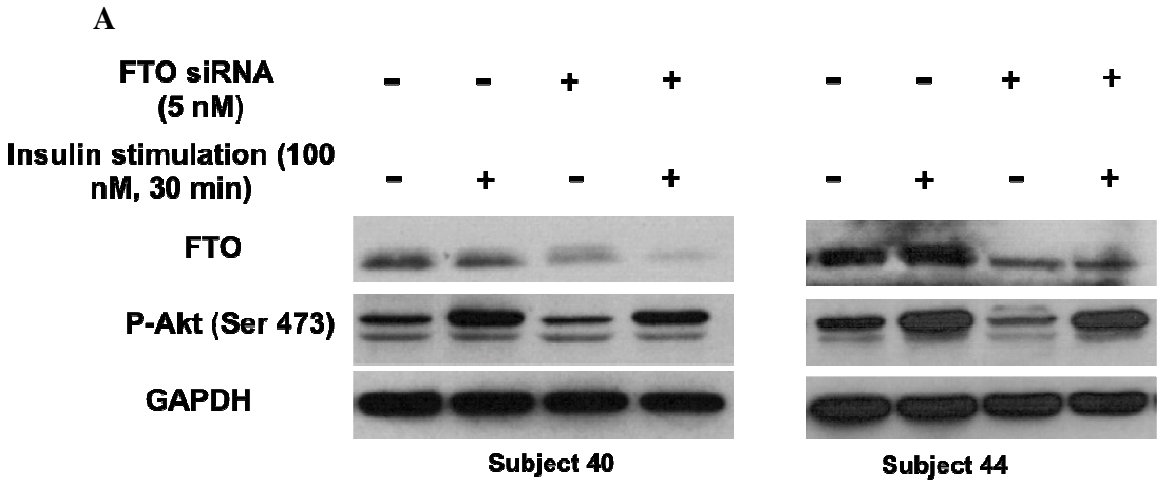


Figure 7.8 (A)Western blots of pAkt (Ser473) and GAPDH in FTO inhibitor (CA) treated myotubes under basal conditions and after insulin stimulation. (B) Graph showing densitometric analysis of p-Akt protein levels normalized against GAPDH protein levels in CA treated primary human myotubes *P < 0.05 versus basal situation. Data are expressed as Mean ± SD.



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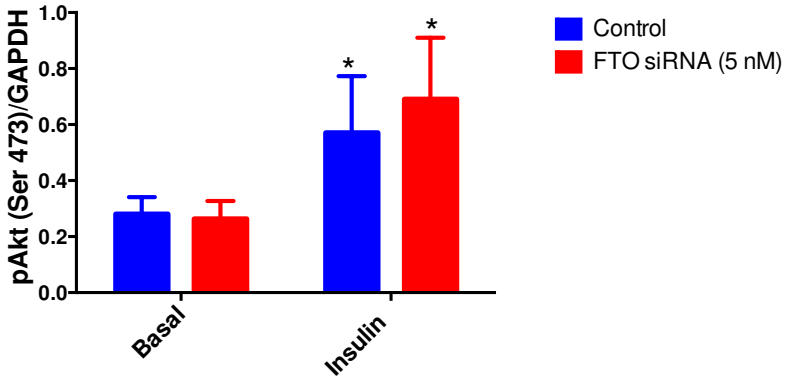

Figure 7.9 (A) Western blots of pAkt (Ser473) and GAPDH in FTO deficient myotubes under basal conditions or after insulin stimulation. Histogram represents *P < 0.05 versus basal situation. B) Graph showing densitometric analysis of p-Akt protein normalized against GAPDH protein levels in FTO knockdown primary human myotubes. Data are expressed as mean ±SD. *P < 0.05 versus basal situation.

7.3.5 m6A methylation level in FTO knockdown and FTO inhibitor (CA) treated primary human myotubes

Lastly, we examined if FTO controls the expression of metabolic-related genes through the dynamic regulation of m6A level on mRNA transcripts. CA treated myotubes showed an increase in the level of m6A modification in total mRNA compared to untreated control (Figure 7.10). Consistent with this result, an increase in the level of m6A methylation in total mRNA was also observed for FTO knockdown myotubes.

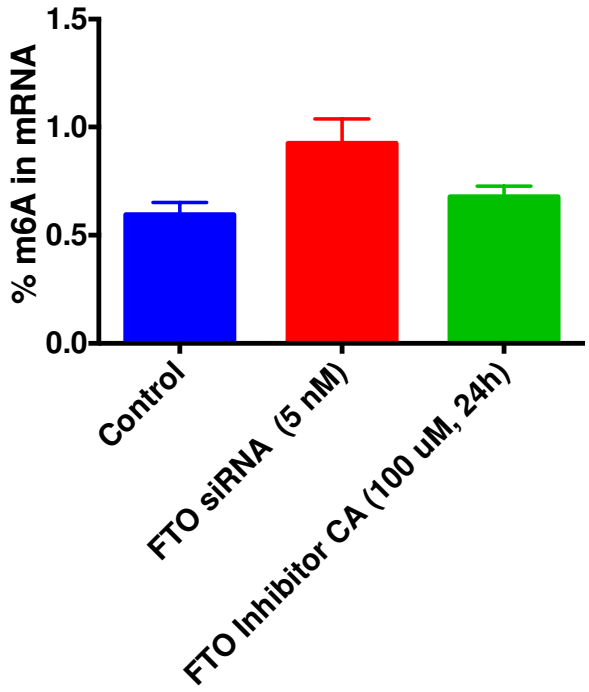

Figure 7.10 FTO inhibition of primary human myotubes by CA (100 µM, 24h) and FTO knockdown (siRNA,5nM) prevents the demethylation of m6A in mRNA

7.4 Discussion

In this study, we demonstrate using two different approaches (siRNA and CA) that both FTO deficiency and FTO inhibition in primary human myotubes enhanced expression of genes involved in fatty acid oxidation and oxidative phosphorylation and decreased expression of genes involved in de novo lipogenesis, suggesting a shift from fat accumulation to fat oxidation in muscle. We also showed that FTO inhibition improved mitochondrial function, especially oxidative metabolism and completely prevented the inhibitory effect of palmitate on basal glucose uptake.

Our results are consistent with the study of Grunnet et al who showed that FTO mRNA expression was negatively associated to basal and insulin stimulated lipid oxidation rate in young and elderly nondiabetic twins(273) and with the study of Bravard et al who showed that increased expression of FTO in human myotubes increased the rate of lipogenesis compared with GFP-overexpressing myotubes(274). In contrast, Church et al reported that a dominant missense mutation in mouse FTO gene (FTO 1367F) upregulated FASN levels suggesting enhanced fat synthesis in muscle. However, there was no increase in fat depots in muscle, therefore a conclusion regarding the association between FTO and lipid storage in muscle could not be drawn in this study(159). We sought to determine whether changes in lipid metabolism might be occurring through PGC1A, a key transcriptional co-activator that regulates mitochondrial biogenesis and oxidative metabolism in muscle. Interestingly, the methylation of the promoter of PGC1A was altered in

muscle of T2D patients(275). We found an increase in PGC1A mRNA levels with FTO inhibition suggesting that PGC1A may be involved in FTO-induced alterations of mitochondrial genes. Similarly, Bravard observed that PGC1A and FTO mRNA levels were regulated in an opposite manner in T2D patients(274). In contrast, Grunnet et al demonstrated a positive correlation between FTO with PGC1A and mitochondrial OXPHOS genes in non-diabetic young and elderly twins(273). Merkestein et al recently showed that PGC1A was upregulated in mice with 2 additional copies of FTO (FTO-4 mice)(276).

FTO inhibition markedly enhanced spare respiratory capacity (SRC) possibly due to higher level of OXPHOS genes such as ATP5J2 and PGC1A. SRC is the amount of extra ATP that can be produced by oxidative phosphorylation at maximal respiratory capacity in response to increased stress or work and is associated with long-term cellular survival and function(277-280). A lack of SRC has been correlated with a variety of pathologies including heart diseases, neuro-degenerative disorders, and cell death in smooth muscle(279, 281, 282). Recently Keuper et al showed that SRC permits primary adipocytes cells to maintain ATP homeostasis under hypoglycemic conditions and may also allow additional metabolic scope for energy dissipation(283). SRC is also increased in skeletal muscles after endurance exercise(172) or as a consequence of caloric restriction. This finding is consistent with a study showing reduced complex I-mediated ATP synthesis in FTO overexpressed C2C12 myotubes(274). Grunnet et al observed faster recovery rates of PCr and Pi in oxidative muscles after exercise in homozygous carriers of the FTO risk allele suggest therefore a higher capacity for aerobic ATP synthesis in

these subjects. However, no association between the FTO genotype and FTO expression in the vastus lateralis muscle was found, therefore the association between FTO expression and mitochondrial efficiency cannot be concluded(284). Many studies in trained individuals have demonstrated that endurance exercise enhanced maximal mitochondrial respiratory capacity(172, 285), fatty acid oxidation(286) and ATP production(287) in muscle, suggesting that FTO may have a role in the regulation of mitochondrial oxidative phosphorylation that contributes to the energy demands of the exercising muscle.

Notably, FTO inhibition had no effect on proton leak but yet an increase in UCP3 mRNA expression was observed. Since proton leak is considered to be physiologically important in energy expenditure as it accounts for 20–25% of basal metabolic rate in rats(243, 288), our results suggest that CA may not have an impact on energy expenditure or may regulate energy expenditure by alternative mechanisms such as futile substrate cycling of fatty acids and triglycerides(75), Ca2+ cycling(11) or protein turnover not measured in this study(289). Our results are consistent with previous finding of unaltered mitochondrial uncoupling in brown adipose tissue of FTO deficient mice(158) but different from that observed in human adipocytes, where FTO deficiency induced mitochondrial uncoupled respiration and UCP1 mRNA expression(290). Evidence for a role of UCP3 in proton leak comes from studies on UCP3 knockout mice, which have lower state 4 respiration (116, 291). It is also shown that obese diet-resistant women have lower mitochondrial state 4 respiration and UCP3 mRNA levels in muscle compared

to diet-responsive subjects(99). Evidence against UCP3 being involved in proton leak includes the finding that fasting increased UCP3 mRNA level in muscle despite a reduced metabolic rate(253). Elevation of UCP3 by a high fat diet in males did not affect mitochondrial proton leak in vivo(123). Our results agree with these findings that UCP3 may not be involved in mitochondrial proton leak. The upregulated UCP3 mRNA expression observed in our study could be a mechanism to sustain increased rates of lipid oxidation via export of FFA anions out of mitochondrial matrix. Under conditions of high fatty acid flux into mitochondria via CPT1B, excessive accumulation of long chain acyl-CoA molecules would be harmful to membranes and sequester CoA, thereby impairing fat oxidation. To prevent these events, upregulation of mitochondrial thioesterase cleaves the acyl-CoA allowing export of the fatty acid anion via UCP3(292). Elevated circulating FFA levels are associated with increased muscle UCP3 expression in fasting, high-fat feeding and obesity, independent of changes in energy expenditure(127, 293)

We next sought to determine whether inhibition of FTO could improve insulin signaling since FTO inhibition resulted in a reduction of de novo lipogensis and increase in fatty acid oxidation. CA was able to alleviate the reduction in glucose uptake caused by palmitate acid, however did not significantly alter basal and insulin-stimulated Akt (Ser473) phosphorylation, suggesting that FTO may not exert its metabolic effects via the IRS1/PI3K pathway. Similarly, Bravard et al observed that silencing FTO in human myotubes did not significantly modify basal Akt (Ser 473) phosphorylation(274). We postulate that FTO may affect other signaling pathways such as AMPK(294)

or p38 MAPK(295) pathways that are activated by exercise.

We also demonstrated that FTO regulates fatty acid metabolism and energy homeostasis by increasing the level of m6A in FTO-targeted genes. Importantly, many of the FTO-regulated genes that were associated with obesity contain m6A sites in their mRNA transcripts. Acaca, Atf6, Bip, Gcdh, Irs1, Perk, and Xbp1 are not only involved in FTO regulated metabolic pathways, they also contain m6A sites(270)

Overall, our results suggest that FTO inhibition by CA improves mitochondria functions especially oxidative metabolism in human skeletal muscle. It also causes an increase in fatty acid oxidation, which is coupled with a decrease in lipogenesis. This likely suggests a shift from lipid accumulation to lipid oxidation. It also improves basal glucose uptake. Hence, our results are consistent with the higher metabolic rate and lower fat mass observed with FTO-/- mouse. Collectively, these findings suggest that pharmacologic inhibition of FTO could be a viable therapeutic strategy for obesity.

Chapter 8 : Conclusions and future directions

Firstly, we evaluated the validity of FAO/WHO/UNU equation and other commonly used prediction equations, Harris Benedict, Mifflin et al and Owen et al for resting energy expenditure (REE) in a sample of healthy Singaporean Chinese men. The results showed that the Owen equation provided a valid estimation of REE in Singaporean Chinese men at a group level. However, the individual errors of the equations were unacceptable high and may have limited utility in making clinical decisions on nutritional requirements. Therefore, in individuals where a precise determination of REE is indicated, measurement by indirect calorimetry instead of the prediction of REE using equations is highly recommended.

Next, we explored the impact of ethnicity on REE and metabolic flexibility (MF) in Chinese, Asian-Indian and Malay men. We have found that Asian Indian men exhibit lower REE than Chinese men for the same body weight. These differences may contribute to the higher prevalence of obesity in this ethnic group. Lower REE in Asian-Indian men may be mediated by smaller size of high metabolic rate organs in the trunk and the brain. To prevent obesity in Asian-Indians, the recommended dietary allowance for energy intake should take into account the lower REE in this ethnic group, and not only on age and body weight as currently recommended by the WHO.

MF, as measured by RQ (iAUC) was independently associated with obesity but not insulin resistance in Chinese, Asian-Indians and Malays. In Malays,

ISI was associated with RQ (iAUC) but the association was completely attenuated by adjustment for obesity. The relationship between MF and obesity is modulated by ethnicity. In lean individuals, Chinese had lower MF than Malays and Indians. However, in overweight/obese individuals, MF was similar between ethnic groups. These ethnic differences seem to relate to differences in the propensity to increase glycolysis, rather than suppression of fat oxidation, after a meal. Thus, altered glycolysis should be considered when examining inter-individual differences in MF. Greater propensity for carbohydrate oxidation and suppression of fat oxidation in lean Asian-Indians and Malays compared to lean Chinese, may predispose them to deplete glycogen stores and ingest more total energy, leading to greater obesity.

We further sought to examine potential inter-individual differences in skeletal muscle characteristics that can explain variability in REE and MF and also the impact of ethnicity on molecular factors affecting skeletal muscle energy metabolism. Variability in REE after adjustment for FFM and fat mass was not associated with mitochondrial respiration in Chinese and Asian-Indian human myotubes. Therefore, skeletal muscle may not have the intrinsic capacity for regulating resting energy metabolism via mitochondrial proton leak. Greater mitochondrial oxidative capacity in lean Asian-Indians compared to Chinese may explain the greater MF observed in the former.

Finally, we characterized the function of FTO in skeletal muscle energy metabolism and fuel utilization. Inhibition of FTO m^6A demethylation activity by CA improved mitochondria function especially oxidative metabolism. CA

also caused an increase in fatty acid oxidation, which is coupled with a decrease in lipogenesis. This likely suggests a shift from lipid accumulation to lipid oxidation. CA was also able to improve basal glucose uptake. FTO regulates fatty acid metabolism and energy homeostasis by modulating the level of m6A in FTO-targeted genes. Collectively, these findings provides string support that pharmacologic inhibition of FTO could be a viable therapeutic strategy for obesity. We hope to extend of our research to animal models. It will be of tremendous clinical interest to determine if FTO inhibitor could bring about similar beneficial metabolic effects in animal models. It is also of tremendous medical interest to map out the RNAs that are the target of FTO's demethylase activity, in order to identify the mRNA targets that give rise to the obesity phenotypes, and to better understand the biological pathways that are regulated by FTO. We hope to build a comprehensive map of FTO-regulated m6A epitranscriptome in human myotubes. In particular, we seek to systematically identify the full range of FTO-targeted gene transcripts/ mRNA that are enriched with m6A sites.

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Appendix A: Manuscripts Submitted and in Preparation

1. Published papers

Tammy Song, Kavita Venkataramanb, Peter Gluckman, Chong Yap Seng, Khoo Chin Meng, Eric Yin Hao Khoo, Melvin Khee Shing Leow, Lee Yung Seng, Tai E. Shyong. Validation of prediction equations for resting energy expenditure in Singaporean Chinese men. Obes Res Clin Pract (2013) http://dx.doi.org/10.1016/j.orcp.2013.05.002

2. Manuscripts Ready to be Submitted

Tammy Song, Kavita Venkataramanb, Peter Gluckman, Chong Yap Seng, Khoo Chin Meng, Michael Chee, Melvin Khee Shing Leow, Lee Yung Seng, Tai E. Shyong, Eric Yin Hao Khoo. Smaller size of high metabolic rate organs explains lower resting energy expenditure in Asian-Indian than Chinese men. Manuscript submitted to American Journal of Clinical Nutrition

Tammy Song, Kavita Venkataramanb, Peter Gluckman, Chong Yap Seng, , Michael Chee, Melvin Khee Shing Leow, Lee Yung Seng, Eric Yin Hao Khoo, Tai E. Shyong, Toh Sue-Anne, Khoo Chin Meng. Metabolic Flexibility Differs among Asian Ethnic Groups in Healthy Lean but not in Overweight or Obese Individuals: the Singapore Adults Metabolism Study (SAMS). Manuscript submitted to Diabetes.

Tammy Song, Kavita Venkataramanb, Peter Gluckman, Chong Yap Seng, Khoo Chin Meng, Michael Chee, Melvin Khee Shing Leow, Lee Yung Seng,

Tai E. Shyong, Toh Sue-Anne. Characterization of FTO (fat mass and obesity associated gene) in human skeletal muscle metabolism and energy homeostasis. Manuscript in preparation for submission to Diabetes.

Appendix B: List of Abstracts and Presentations

Tammy Song, Kavita Venkataramanb, Peter Gluckman, Chong Yap Seng, Khoo Chin Meng, Eric Yin Hao Khoo, Melvin Khee Shing Leow, Lee Yung Seng, Tai E. Shyong. Validation of prediction equations for resting energy expenditure in Singaporean Chinese men. Poster presentation in Yong Loo Lin School of Medicine 2nd Annual Graduate Scientific Congress 2012.

TLL Song, et al. Resting Energy Expenditure in Singaporean Chinese. Annals of the Academy of Medicine, Singapore (Proceedings of the 1st Singapore Health & Biomedical Congress 2010), November 2010, Vol. 39 (Suppl) No. 1, S173.

Tammy Song, Kavita Venkataramanb, Peter Gluckman, Chong Yap Seng, Khoo Chin Meng, Michael Chee, Melvin Khee Shing Leow, Lee Yung Seng, Tai E. Shyong, Eric Yin Hao Khoo. Smaller size of high metabolic rate organs explains lower resting energy expenditure in Asian-Indian than Chinese men. Oral presentation in Yong Loo Lin School of Medicine 2nd Annual Graduate Scientific Congress 2012.

Tammy Song, Kavita Venkataramanb, Peter Gluckman, Chong Yap Seng, , Michael Chee, Melvin Khee Shing Leow, Lee Yung Seng, Eric Yin Hao Khoo, Tai E. Shyong, Toh Sue-Anne, Khoo Chin Meng. Metabolic Flexibility Differs among Asian Ethnic Groups in Healthy Lean but not in Overweight or

Obese Individuals: the Singapore Adults Metabolism Study (SAMS). Manuscript in preparation for submission to Diabetes Poster presentation at American Diabetes Association: 74th Scientific Associations, 2014, San Francisco, California.